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CHARACTERISTICS OF THE ADRENAL ASCORBIC ACID RESPONSE TO ADRENOCORTICOTROPHIC HORMONE (ACTH) IN THE RAT[1]

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ABSTRACT

The rate of ascorbic acid release from the adrenals of intact rats in response to endogenous ACTH secretion has been systematically determined following unilateral adrenalectomy, sham-adrenalectomy, cannulation of femoral artery and vein, sciatic nerve stimulation, and the subcutaneous administration of L-epinephrine. The adrenal activation in response to ACTH administered to hypophysectomized rats has also been investigated. Examination of the kinetics of the adrenal ascorbic acid response to either endogenous or exogenous ACTH reveals that regardless of the type of experimental procedure applied as a stress the adrenal ascorbic acid changes occur at a uniform rate. Calculation of the relative rates of adrenal ascorbic acid depletion and repletion indicates that the rate of ascorbic acid loss from the rat adrenal is about 120 times more rapid than the rate of recovery. A linear relationship between the log dose and response can be reliably demonstrated for doses of ACTH up to 5 μg. when the usual one hour interval is employed between injection of ACTH and adrenal gland removal. Doses of ACTH greater than 5 μg., or severe stresses (i.e. unilateral adrenalectomy) require longer time intervals to attain maximal adrenal ascorbic acid depletion. The range of the dose-response curve for adrenal ascorbic acid depletion may be extended appreciably by using a two-hour interval between ACTH injection and removal of the second adrenal gland. Four hours after unilateral adrenalectomy an increase in weight of the remaining adrenal can be demonstrated. This rapid increase in adrenal weight is not the result of a true tissue hypertrophy but is the result of an accumulation of fluid in the remaining adrenal. The reliability and limitations of the adrenal ascorbic acid index for quantitative ACTH estimations are discussed.

INCREASED adrenocortical activity is normally associated with a reduction in the concentration of ascorbic acid in the adrenal gland. Although the depletion of adrenal ascorbic acid has been widely used as an

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indirect index for the quantitation of ACTH secretion, virtually nothing is known of the relationship between adrenocortical hormone synthesis and ascorbic acid metabolsim. Until specific detailed information is obtained, with respect to the magnitude and the duration of the adrenal ascorbic acid changes which result from activation by ACTH, the usefulness and reliability of this index will remain in question.

A somewhat detailed study has been made of the adrenal response to ACTH in order to characterize the dynamics involved in this adrenal response. No general concepts or pattern of the early ascorbic acid response to ACTH could be established from the data of earlier reports (1) since these studies were confined to adrenal gland analyses at 20 minutes and at one hour. In the present investigation, the time-course of the changes in adrenal ascorbic acid have been charted in response to either endogenous or exogenous ACTH in order to clearly establish the kinetics of this response.

EXPERIMENTAL METHODS

Animals

Male rats of the Sprague-Dawley strain, whose body weight ranged from 200–300 gm., were used in this investigation.

Hypophysectomized animals were obtained from a commercial breeder. They were used 2–4 days postoperatively and were carefully examined for completeness of hypophysectomy at the end of the experiments.

Procedures

All animals were anesthetized with sodium pentobarbital (Nembutal), 5 mg./100 gm. body weight, given intraperitoneally at the time of the experiment.

Unilateral adrenalectomy was performed via the dorsal approach. The same procedure was used for the sham-adrenalectomy, except for the extirpation of the adrenal.

Cannulation of the femoral artery and vein was performed just below the inguinal ligament. The femoral nerve was severed and the blood vessels were eventually tied off.

Sciatic nerve stimulation was carried out in the following manner: the sciatic nerve was exposed, dissected free of fascia, and the peripheral end cut. Wire electrodes were placed on the nerve and stimulation carried out by means of a square wave stimulator connected to a rotary drum which permitted the animal to receive the stimulation for only 15 seconds of every minute. The parameters of stimulation were: frequency of 100/sec.; pulse duration of 1 msec.; and voltage of 7 volts.

L-epinephrine was freshly prepared from the bitartrate salt and made up in a 20 mg.% solution of glutathione in saline to prevent rapid oxidation. A subcutaneous injection of 50 μg. in 0.5 ml. solution was given to each experimental animal under anesthesia.

ACTH solutions were freshly prepared each day and kept refrigerated when not in use. ACTH (Upjohn, 1 U.S.P. unit/mg.) lyophilized powder was diluted with saline so that the dose administered was contained in 0.5 ml. solution. Intravenous injections were given into the femoral vein in doses of 2, 5 and 20 μg. per animal. The duration of these injections was approximately 10 seconds.

Ascorbic Acid Analysis and Adrenal Weight

The adrenals were extirpated via the dorsal approach. They were cleaned and rapidly weighed on a torsion balance and placed in 6% trichloracetic acid for subsequent as-

corbic acid analysis by the method of Roe and Kuether (2). Some of the adrenals were dried in an oven at 105° C for 48 hours in order to obtain the dry weights.

RESULTS

Unilateral Adrenalectomy

Unilateral adrenalectomy is the best procedure to use in order to observe the progressive changes that occur in the adrenal ascorbic acid after stress since the excised gland not only serves as a control but its removal also acts as the stress stimulus. The second gland was removed at varying periods of time after removal of the control gland and the changes in both wet and dry weight and ascorbic acid concentration were determined.

Following unilateral adrenalectomy in the rat, it has been found that the fall in adrenal ascorbic acid begins with great rapidity and can be detected as early as 2 min. after the stress (Table 1). The fall continues for about four hours; thereafter the concentration in the gland begins to increase, requiring 30 hours before the initial level is restored. The data in Table 1 show that if a comparison is made between the percentage change in total ascorbic acid in the gland and the concentration of adrenal ascorbic acid following removal of one adrenal, a significant difference is observed between these parameters during the recovery phase. Total adrenal ascorbic acid returns more rapidly to normal levels than is apparent from a determination of ascorbic acid concentration. This is due to the marked increase in adrenal weight, evident after the fourth hour, which continues until a maximum increase is reached 12 hours following removal of one adrenal. Reference to Table 2 will show that the increase in wet weight of the remaining adrenal is not associated with an increase in its dry weight. The rapid increase in adrenal weight is not the result of a true hypertrophy of

TABLE 1. EFFECT OF UNILATERAL ADRENALECTOMY ON ADRENAL ASCORBIC ACID AND ADRENAL WEIGHTS OF INTACT RATS

No. of expts.	Time after unil. adrex.	% change in adrenal ascorbic acid		Wt. right adrenal
		Total	Conc. ±S.E.*	Wt. left adrenal
6	0		+ 1.5±0.81	
6	2 min.	− 8.6	− 2.2±1.39	0.93
6	5 min.	−16.4	− 5.7±1.80	0.88
6	10 min.	−25.7	−14.3±2.40	0.87
6	15 min.	−24.8	−20.7±0.86	0.95
6	30 min.	−30.4	−26.4±2.32	0.94
9	1 hour	−37.9	−34.5±2.28	0.95
6	2 hours	−48.4	−44.9±3.56	0.93
6	4 hours	−47.1	−47.0±5.87	1.00
6	6 hours	−40.3	−39.4±3.63	0.98
6	9 hours	−19.9	−26.3±2.09	1.08
10	12 hours	−10.8	−20.6±3.21	1.12
8	15 hours	− 5.5	−15.2±2.80	1.12
10	18 hours	− 6.1	−13.1±3.14	1.09
6	21 hours	− 0.5	−10.4±3.80	1.12
6	24 hours	− 3.5	−10.2±3.85	1.07
7	30 hours	− 9.1	− 4.5±3.25	1.13

* Standard Error of the Mean.

TABLE 2. ADRENAL WEIGHT CHANGES FOLLOWING UNILATERAL ADRENALECTOMY OF INTACT RATS

Time after unil. adrex.	Adrenal weight			
	Dry weight		Wet weight	
	No. expts.	Right / Left	No. expts.	Right / Left
0–2 hrs.	24	0.97	69	0.94
4 hrs.	10	0.87	16	0.98
6 hrs.	13	0.95	19	1.02
12 hrs.	7	0.96	17	1.11

adrenal tissue, but is primarily due to an accumulation of fluid in the remaining adrenal.

If the per cent ascorbic acid change in the adrenal at any time after removal of one adrenal is plotted against the logarithm of time, a linear relationship is shown to exist. Figure 1 indicates that after unilateral adrenalectomy, this adrenal response is a direct function of the log time after operation. This relationship may be expressed in the form of an equation in which the per cent adrenal ascorbic acid decrease can be calculated at any instant in time (minutes). This equation is:

$$\% \text{ A.A. Decrease} = 23 \log \text{time} - 8$$

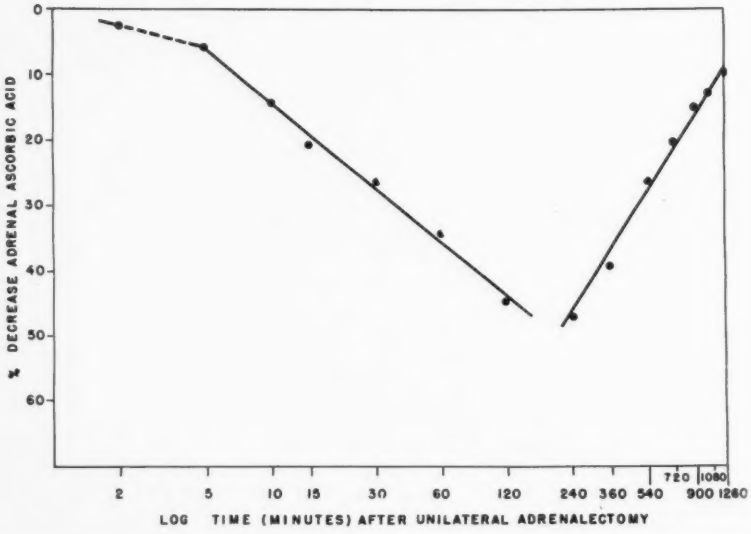

FIG. 1. Per cent adrenal ascorbic acid changes plotted against logarithm of time following unilateral adrenalectomy of intact rats. Each point represents the mean of 6–10 rats.

The derivative of this equation is:

$$\frac{dy}{dt} = \frac{10.0}{t}$$

Similarly, the equation for the recovery phase of adrenal ascorbic acid may be obtained from figure 1:

$$\% \text{ A.A. Decrease} = -56 \log \text{time} + 180$$

Differentiating, we get:

$$\frac{dy}{dt} = \frac{-24.3}{t}$$

With this information, the adrenal ascorbic acid change at any time after removal of one adrenal can be calculated, as well as the change in rate of this adrenal response.

Other Stresses

The adrenal ascorbic acid changes which occur following such experimental procedures as cannulation of blood vessels, stimulation of a nerve, sham operations, and the injection of epinephrine are presented in Table 3. A comparison may be made between the effects of endogenously secreted ACTH on adrenal ascorbic acid depletion with the effect of a single intravenous injection of 20 μg. ACTH into hypophysectomized rats. This amount of ACTH results in a maximal depletion of adrenal ascorbic acid (infra, Table 4) and is therefore used as the standard for comparison. Figure 2 illustrates the straight lines obtained when the 20 μg. ACTH injection data are fitted by the method of least squares. The 5 per cent confidence limits for these lines are represented by the dotted lines. The general equation for the linear function representing the adrenal ascorbic acid depletion phase is:

$$\% \text{ A.A. Decrease} = 21.0 \log \text{time} - 4.2$$

whereas the equation for the recovery phase is:

$$\% \text{ A.A. Decrease} = -36.8 \log \text{time} + 118.5$$

When the data which were obtained from endogenously released ACTH (Table 3) are plotted and superimposed on a single graph (Fig. 2), it becomes apparent that the rate of adrenal ascorbic acid response in the intact rat to these experimental procedures is quite similar to that observed following the single intravenous injection of ACTH into hypophysectomized rats. Figure 2 illustrates that most of the observed values lie within the 5% confidence limits; therefore it may be concluded that the rate of adrenal ascorbic acid response is uniform regardless of the type of "stress" employed.

TABLE 3. EFFECT OF VARIOUS EXPERIMENTAL PROCEDURES ON ADRENAL ASCORBIC ACID OF INTACT RATS

Time after stress	Cannulation operation			L-epinephrine (0.02 mg. /100 gm. B.W.) sc			Sham adrex.			Sciatic n. stim.		
	No. of expts.	Adrenal ascorbic acid		No. of Expts.	Adrenal ascorbic acid		No. of expts.	Adrenal ascorbic acid		No. of expts.	Adrenal ascorbic acid	
		Mg. % ± S.E.	% change ± S.E.*		Mg. % ± S.E.	% change ± S.E.*		Mg. % −S.E.	% change −S.E.*		Mg. % −S.E.	% change −S.E.*
Controls	134	478 ± 5.10	0.0 ± 1.06	134	478 ± 5.10	0.0 ± 1.06	134	478 ± 5.10	0.0 ± 1.06	134	478% 5.10	0.0 ± 1.06
15 min.	5	380 ± 18.5	−20.5 ± 4.88	6	438 ± 27.7	− 8.6 ± 6.33	10	374 ± 34.8	−21.8 ± 7.28	10	398 ± 16.9	−16.7 ± 4.25
30 min.	8	338 ± 8.6	−29.3 ± 2.54	6	424 ± 24.4	−11.3 ± 5.77	5	329 ± 14.7	−31.2 ± 3.09	4	354 ± 22.2	−26.0 ± 6.27
1 hour	10	330 ± 18.8	−31.0 ± 5.72	6	343 ± 27.3	−28.2 ± 7.97	5	290 ± 27.0	−39.4 ± 5.64	4	308 ± 18.8	−35.6 ± 6.10
2 hours	15	277 ± 10.1	−42.1 ± 3.66	11	313 ± 15.1	−34.5 ± 4.83	5	277 ± 6.9	−42.1 ± 1.44	4	312 ± 13.2	−34.7 ± 4.24
4 hours	10	326 ± 16.6	−32.0 ± 5.12	12	300 ± 10.9	−37.3 ± 3.64	5	350 ± 31.8	−26.8 ± 6.67	4	355 ± 12.9	−25.8 ± 3.64
6 hours	10	334 ± 11.9	−30.2 ± 3.57	6	371 ± 6.7	−22.4 ± 1.80						
12 hours	5	477 ± 10.7	0.0 ± 2.24	6	428 ± 24.7	−10.4 ± 5.77						

* Standard Error of the Mean.

TABLE 4. EFFECT OF A SINGLE INTRAVENOUS INJECTION OF ACTH ON THE ADRENAL ASCORBIC ACID OF HYPOPHYSECTOMIZED RATS

Time after injection	Amount of ACTH injected								
	2 μg.†			5 μg.†			20 μg.†		
	No. of expts.	% change in adrenal ascorbic acid ± S.E.*	Adrenal wt. Right / Left	No. of expts.	% change in adrenal ascorbic acid ± S.E.*	Adrenal wt. Right / Left	No. of expts.	% change in adrenal ascorbic acid + S.E.*	Adrenal wt. Right / Left
Controls	17	+ 2.4 ± 0.73							
2 mins.				6	− 6.5 ± 1.29	0.92	6	− 7.9 ± 1.67	0.98
5 mins.	5	− 7.9 ± 3.69	0.94	6	−15.2 ± 2.33	0.95	6	−13.8 ± 3.79	0.97
10 mins.				7	−15.1 ± 2.32	0.95	5	−15.5 ± 1.49	0.99
15 mins.	5	−18.9 ± 1.13	0.95	6	−18.6 ± 2.15	0.99	5	−15.0 ± 2.20	0.91
30 mins.	5	−23.5 ± 2.50	0.98	6	−27.6 ± 2.85	0.90	6	−25.3 ± 3.05	0.97
1 hour	7	−22.8 ± 2.28	0.91	6	−30.1 ± 2.46	0.93	6	−32.9 ± 2.19	0.96
2 hours	5	−22.1 ± 4.22	0.97	6	−29.9 ± 2.42	0.97	6	−39.2 ± 3.06	0.96
4 hours	5	−16.9 ± 2.25	0.95	6	−22.3 ± 1.64	0.95	5	−33.0 ± 4.43	0.95
6 hours				6	−18.4 ± 2.72	0.94	5	−24.6 ± 1.93	0.94
12 hours	5	− 6.6 ± 1.75	0.95	6	− 8.4 ± 1.83	0.93	5	− 9.3 ± 2.19	0.97
18 hours				6	− 7.2 ± 1.79	0.94	4	− 8.3 ± 1.19	0.96
24 hours				6	−15.0 ± 1.57	1.00	5	−14.6 ± 2.25	0.94
30 hours				6	−11.7 ± 1.74	0.94	5	− 8.7 ± 4.81	0.86

* Standard error of the mean.
† Total dose per 250 gm. rat.

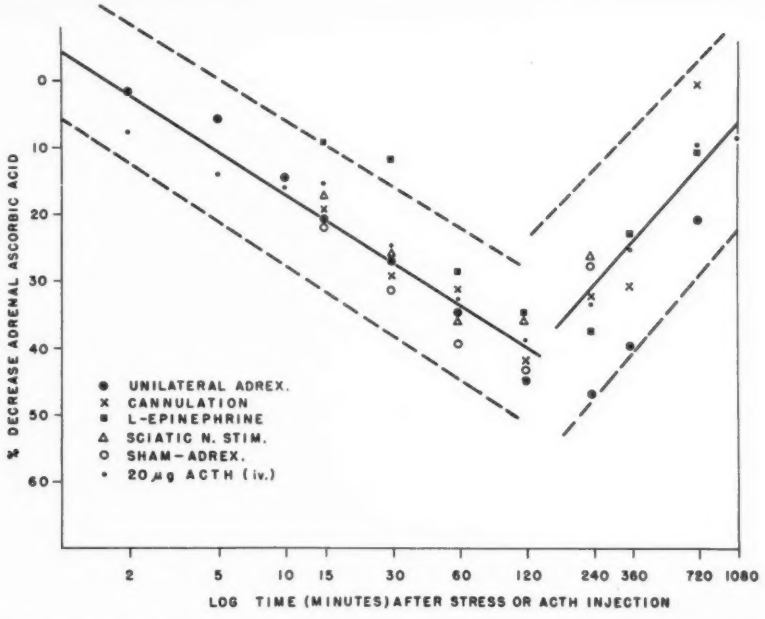

FIG. 2. Per cent adrenal ascorbic acid changes plotted against logarithm of time following various experimental procedures in intact rats or after a single intravenous injection of ACTH into hypophysectomized rats. The solid line is obtained by fitting the 20 µg. ACTH injection data by the method of least squares whereas the dotted lines represent the 5% confidence limits for this line. The effects of endogenously released ACTH on adrenal ascorbic acid are superimposed on this graph.

Infusion of ACTH

Injection of ACTH into hypophysectomized animals permits more exact control of the magnitude and duration of the ACTH stimulus to the adrenal gland, therefore the adrenal ascorbic acid response to specific quantities of ACTH may be determined. Experiments were performed on hypophysectomized rats to determine the adrenal response in time to various doses of a rapid single intravenous injection of ACTH.

Table 4 gives the adrenal ascorbic acid depletion obtained when 2, 5, and 20 µg. ACTH are injected intravenously into hypophysectomized rats. The similarity of the resulting adrenal response pattern to that observed following stress is illustrated in Figure 3. It is evident that at the end of one hour a difference may be detected between the adrenal ascorbic acid depletion elicited by 2 µg. ACTH as compared to 5 µg. ACTH. On the other hand two hours are required in order to detect a differential response between the larger doses of 5 and 20 µg. ACTH.

It will be noted (Table 4) that no increase in adrenal weight occurs even

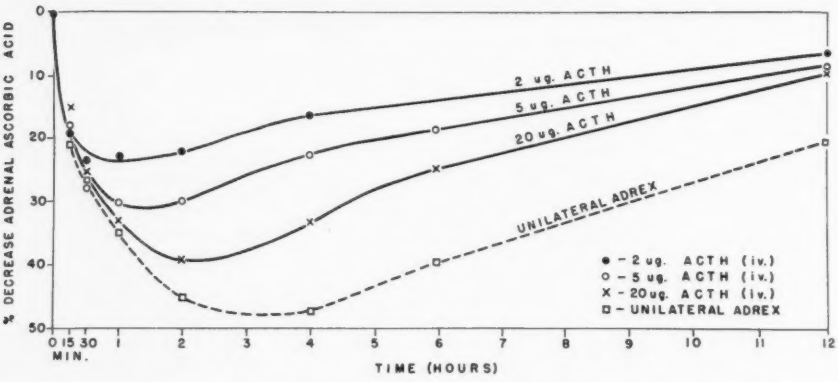

FIG. 3. The pattern of adrenal ascorbic acid changes in time following a rapid single intravenous injection of ACTH into hypophysectomized rats as compared to the effect of a prolonged secretion of ACTH following unilateral adrenalectomy of intact rats.

after the injection of 20 μg. ACTH into hypophysectomized rats. In contrast to what is observed following unilateral adrenalectomy, no adrenal weight change was produced with any amount of ACTH injected intravenously as a single dose into hypophysectomized animals, even though the ascorbic acid changes were comparable. This increase in weight may be dependent upon the different effects produced by a limited or continuous stimulation of the adrenal cortex by ACTH.

DISCUSSION

The present investigation has demonstrated conclusively that regardless of the type of experimental procedure applied as a stress, the adrenal ascorbic acid changes occur at a uniform rate that can be linearly related to the logarithm of time. This uniformity of response has not been reported previously and is of significance when this index is used as evidence of ACTH secretion. Moreover, the kinetics of ascorbic acid depletion must be established in order to obtain a better understanding of the relationship between hormonal synthesis and ascorbic acid metabolsim in the adrenal gland.

Although the ascorbic acid depletion index has been used successfully in the hypophysectomized rat for the quantitation of ACTH in samples of blood, its use in the intact rat must be tempered and qualified. The concentration of adrenal ascorbic acid in the intact rat does not necessarily reflect the blood concentration of ACTH at that particular time as indicated by simultaneous studies of the blood level of ACTH and the ascorbic acid changes in the adrenals of the intact rat (3). The results of the present study on the effect of a single intravenous injection of ACTH in the hypophysectomized rat indicate that once ACTH has activated the adrenal, its effect on the concentration of ascorbic acid in this gland continues long

after the ACTH has disappeared from the blood. The rate of ascorbic acid depletion is the same regardless of whether this is brought about by a single burst of ACTH secretion (exemplified by a single injection into hypophysectomized rats) or by a prolonged secretion as normally occurs in the intact rat. No indication of the duration of ACTH secretion or of the total quantity of hormone secreted, can be obtained by the use of this index in the intact rat. On the other hand, the adrenal ascorbic acid depletion index for ACTH assay is quantitatively reliable when hypophysectomized rats are employed under standardized conditions as in a previously reported cross-circulation procedure (4). Under these conditions the magnitude of the fall in the ascorbic acid content of the hypophysectomized rat adrenal provides an accurate estimate of the amount of ACTH in the infused sample of blood at that particular time.

In order to interpret the prolonged, yet quantitative, adrenal response to a single intravenous injection of ACTH in the hypophysectomized rat, the studies of Sonenberg *et al.* (5), using ACTH labeled with I^{131}, would appear to offer some explanation for this phenomenon. By means of adrenal radioautographs, these investigations showed that ACTH was taken up by the adrenal and then released slowly, in a logarithmic time sequence. From these results, it may be postulated that ACTH is "trapped" in the adrenal gland and continues to stimulate the depletion of ascorbic acid; the duration of this response may depend upon the logarithmic decline of ACTH in the adrenal gland. In view of the extremely short half life of ACTH in the blood (6), a persistent activation at the site of the adrenal is implied by the continuation of adrenal activity long after the ACTH has presumably disappeared from the circulation.

The adrenal ascorbic acid changes occur at a uniform rate when these changes are plotted against the logarithm of time. Although the significance of this linear log time factor is not completely understood, its occurrence in nature is rare. Stacy (7), in a study of tissue elasticity, reasoned that the logarithm of time relationship could best be interpreted to indicate the existence of two interdependent exponential reactions. This analysis may be utilized in the present study since it is well known that in the rat adrenal both ascorbic acid depletion as well as accumulation can occur. The ascorbic acid concentration in the rat adrenal will therefore be the net algebraic sum of these two exponential reactions which are going on simultaneously. Evidence to support this hypothesis will be found in Figure 4 which demonstrates the pattern obtained when the logarithm of the ascorbic acid remaining in the adrenal is plotted agianst time after unilateral adrenalectomy. The straight line relationship which occurs in the first 15 minutes indicates that ascorbic acid depletion is occurring predominantly or exclusively during this time, whereas the linear function which is obtained during the last 15 hours of this study, indicates that reaccumulation of ascorbic acid in the adrenal is proceeding virtually unopposed. Dur-

ing the interval between 15 minutes and 15 hours, both processes are probably going on simultaneously. When the relative rates of adrenal ascorbic acid decrease and recovery are calculated, it is found that the rate of ascorbic acid loss from the adrenal of the rat is approximately 120 times more rapid than the rate of recovery.

In the original Sayers *et al.* ACTH assay technique (8), the period of one hour after application of a stress or the injection of ACTH had been selected as the time at which an estimate could be made of the quantity of ACTH responsible for adrenal ascorbic acid depletion. These investigations

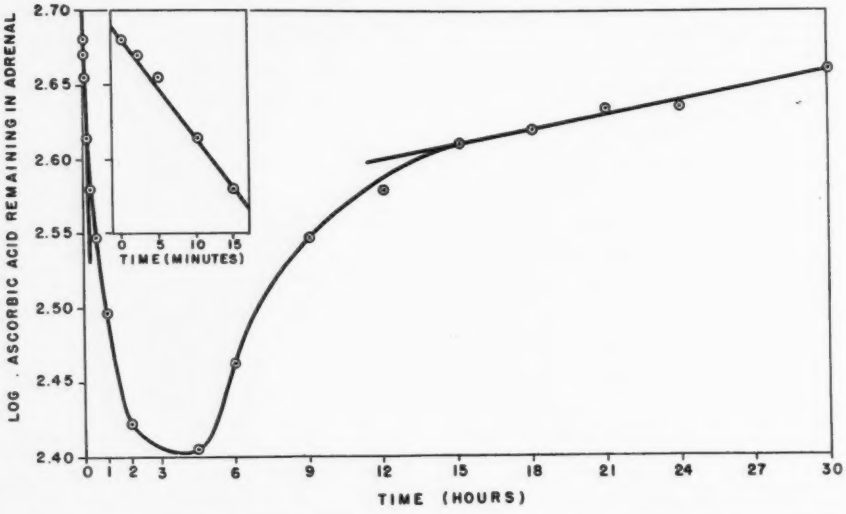

FIG. 4. Pattern of adrenal ascorbic acid changes obtained when the logarithm of ascorbic acid remaining in adrenal is plotted against time after unilateral adrenalectomy. Insert is a magnified view of the changes occurring in the first 15 minutes.

demonstrated a linear dose-response correlation for doses of ACTH up to 5 μg. This is in agreement with the results reported herein, for Table 4 and Figure 3 demonstrate that 5 μg. ACTH produces a maximal effect at the end of one hour and that greater quantities of ACTH cannot be distinguished within this one hour interval. However, doses of ACTH greater than 5 μg, or severe stresses such as unilateral adrenalectomy, require longer time intervals to attain maximal adrenal ascorbic acid depletion, and if one continues to follow the adrenal ascorbic acid response for a longer period of time, changes in the higher dose range become manifest. Significant further depletion of adrenal ascorbic acid can be detected when the adrenal glands are analyzed two hours (rather than at the customary one hour interval) after either unilateral adrenalectomy or a 20 μg intravenous injection of ACTH. The range of the dose-response curve for ad-

renal ascorbic acid depletion may therefore be extended appreciably by using a two hour interval between ACTH injection and removal of the second adrenal gland.

Adrenal hypertrophy following unilateral adrenalectomy has been reported by Weisz (9). He observed this effect at 24 and 48 hours after removal of one adrenal, whereas in the present investigation, adrenal enlargement is evident 4 hours after stress. Ingle (10) has also reported adrenal weight increases 12 hours after subjecting rats to a continuous work test. A comparable adrenal enlargement could be produced in hypophysectomized rats by the subcutaneous administration of large amounts of ACTH throughout an 8 hour period. In the present study, hypophysectomized rats failed to show an increased adrenal weight following large single doses of intravenously administered ACTH. Adrenal enlargement is probably the result of a continuous stimulation of the adrenal by high blood levels of ACTH brought about either by prolonged secretion of ACTH from the pituitary of intact animals or by continued absorption of subcutaneous ACTH in hypophysectomized rats.

In conclusion, we must reemphasize the limitations of the adrenal ascorbic acid depletion index as a criterion of pituitary-adrenocortical secretion (11) in the intact animal. Caution should always be used in the interpretation of results obtained in intact animals exposed to a procedure which is known to result in ACTH release since no quantitative information may be deduced from this index with regard to either the amount or the duration of hormone secretion.

Acknowledgment

We wish to express our appreciation to Dr. Harold Lamport for many helpful suggestions concerning the mathematical analysis and interpretations.

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THYROXINE ANALOGUES AND CHOLESTEROL METABOLISM: THE RELATIVE EFFECTS OF VARIOUS THYROXINE DERIVATIVES ON GROWTH, OXYGEN CONSUMPTION AND TISSUE CHOLESTEROL CONCENTRATIONS IN THE RAT

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ABSTRACT

A number of thyroxine analogues have been tested for their relative effects on tissue cholesterol concentrations, oxygen consumption, and growth in the hypercholesterolemic rat. Of the compounds studied, diiodothyroacetic (Diac) and diiodothyroformic acid (T_2 FA) appear to have the greatest effect in reducing tissue cholesterol concentrations without affecting growth or basal metabolism. Other derivatives such as tri- and tetraiodothyroacetic acids, triiodothyropropionic acid and tetraiodothyroformic acid have been shown by others to exert some preferential effect on tissue cholesterol concentrations. However, when compared on an equal molar basis in the present study, these compounds were not nearly as effective as Diac and T_2 FA in lowering cholesterol levels.

SINCE elevated plasma cholesterol concentrations are frequently observed in persons predisposed towards atherosclerosis, it is important to consider any compound which will lower tissue concentrations of cholesterol. Thyroid extract or thyroxine administration will lower plasma cholesterol concentrations in hypothyroid and euthyroid subjects (1–3), but such substances probably cannot be considered as potential therapeutic agents in atherosclerosis because of their general metabolic effects. Therefore, this study has been directed toward finding a thyroxine analogue(s) which will lower tissue cholesterol concentrations without influencing basal metabolism, growth or endogenous thyroid function.

Several thyroxine derivatives have been proposed as compounds fulfilling these requirements. For example, tri- and tetraiodothyroacetic acid administration produced a reduction in plasma cholesterol levels in myxedematous subjects and in euthyroid persons with hypercholesterolemia (4–8). However, the effect could not be maintained and the cholesterol concentrations returned to pretreatment levels. Duncan and Best (9) found that when administered in amounts having equal goiter prevention activity, tetraiodothyroformic acid was more effective than thyroxine in lowering plasma and liver cholesterol concentrations in thiouracil fed rats.

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Schaefer and Aldersberg (10) and Sachs and Arons (11) have observed a significant decrease in the serum cholesterol values of human subjects receiving daily oral doses of 1 to 8 mg. of triiodothyropropionic acid. The latter authors also found that triiodothyropropionic acid produces an increase in the serum protein bound iodine concentration and basal metabolism of hypothyroid persons and an elevation of the serum protein bound iodine concentration in euthyroid subjects. The implications of the protein bound iodine data are not readily apparent.

Since a systematic comparison of the relative potencies of a number of thyroxine derivatives on tissue cholesterol concentrations has apparently never been made, we have performed such an experiment (12). Our initial objective has been to determine which analogues show the greatest preferential effect on cholesterol metabolism without affecting other biological parameters such as growth and basal metabolism.

METHODS

Weanling male Albino rats (Holtzman) were housed by pairs in wire mesh-bottomed cages in an air conditioned animal room maintained at approximately 25° C. They were fed either a Purina chow or purified high fat-high cholesterol diet containing cholic acid, *ad libitum*. The latter type of ration has been shown to produce hypercholesterolemia in the rat (13) and has the composition shown in Table 1.

The thyroxine analogues studied[1] were prepared for injection by dissolving them in 0.1 N NaOH, and adjusting the final pH to 8 to 9. Physiological saline solution adjusted to the same pH was used in the control chow and high fat diet groups. Intraperitoneal injections of 0.2–0.5 ml. of these solutions were made every other day at a concentration of 0.5 micromoles per 100 gm. body weight per day.

At the termination of the injection period, the rate of oxygen consumption was determined for each animal in a closed-system calorimeter maintained at 26° C.[2] These measurements were always made between 9 A.M. and 2 P.M. The animals were allowed to equilibrate in this system for six minutes, after which three-minute determinations of the oxygen consumption rate were repeated until successive triplicate values were obtained. Oxygen consumption was expressed as liters of O_2 consumed per hour per M^2 of body surface area.

After completion of the oxygen consumption measurements, the animals were anesthetized with ether, and heparinized blood samples were obtained by cardiotomy for plasma cholesterol determinations. The livers were removed *in toto* and weighed. Approximately 0.5 gm. samples weighed to an accuracy of ± 5 mg. were frozen and ground in 1–2 ml. of Bloor's reagent (three parts absolute ethyl alcohol, one part absolute diethyl ether), and the final volume made up to 30 ml. with Bloor's reagent. The animal carcasses were further necropsied for gross abnormalities.

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[1] The authors are very grateful to Drs. Robert L. Kroc and A. Wayne Ruddy of the Warner-Lambert Research Institute for supplying generous amounts of 3,5,3′-triiodothyropropionic acid, 3,5,3′,5′-tetraiodothyropropionic acid, 3,5-diiodothyropropionic acid and 3,5-diiodo-4-(4′-hydroxy-3′5′-diiodophenoxy) benzoic acid; and to Drs. Dorsey Holtkamp and A. E. Hemming of the Smith, Kline & French Laboratories for equally generous amounts of D-3,5,3′-triiodothyronine, 3,5,3′-triiodothyroethylamine and 3,5-diiodo-4-(4′-hydoxy-3′,5′-diiodophenoxy) benzoic acid.

[2] The authors are indebted to Dr. Richard Doisy for the use of this instrument.

TABLE 1. PERCENTAGE COMPOSITION OF THE PURIFIED HIGH FAT-HIGH CHOLESTEROL DIET

	Gm. %
Casein	16.0
Glucose	50.0
Lard	29.0
Salts[1]	3.0
Cholesterol	1.0
Cholic acid	0.5
Vit. A Concentrate[2]	0.3
Vit. D Concentrate[3]	0.3
Alpha tocopherols	0.03
Water soluble vitamins	**Mg. %**
Thiamin	0.375
Riboflavin	0.470
Pyridoxine	0.375
Nicotinic acid	1.875
Pantothenic acid	1.875
Choline chloride	187.500
Folic acid	0.375
Vit. B_{12}	0.015
Biotin	0.094
Inositol	93.705

[1] Phillips & Hart Salt Mixture.
[2] 200,000 I.U. Vit. A/gm.
[3] 20,000 I.U. Vit. D/gm.

Plasma cholesterol concentrations were determined on 0.5–1.0 ml. of plasma, using the Sperry-Webb modification of the Schoenheimer Sperry method (14). Liver cholesterol concentrations were determined by the same method on 3 ml. of the Bloor extract, and liver lipids were expressed as total solids (mg. %) in the Bloor extract.

RESULTS

In the first experiment, 88 animals were distributed into eleven groups of 8 rats each. The average mean weight for each group was the same. Group 1 received the Purina chow diet, and the other ten groups were fed the high fat diet (HFD). At the end of two weeks, plasma cholesterol concentrations were measured in tail vein samples obtained from four animals each in Groups 1 and 2. These values averaged 436 and 55 mg. % respectively for the HFD and chow diet groups. Following this successful production of hypercholesterolemia in animals receiving the high fat diet, injections of the thyroxine derivatives listed in Table 2 were started in Groups 3–11. After a two-week injection period, individual oxygen consumption rates were measured, and plasma and liver samples were collected for cholesterol and total lipid determinations. These data are summarized in Table 2.

Since the object of this study was to determine which analogues diminish tissue cholesterol concentration without affecting oxygen consumption and growth, thyroxine (T_4), triiodothyronine (T_3), and triiodothyroacetic acid (Triac) can be eliminated as compounds of immediate interest (Table 2). These compounds produced a significant growth depression and increase in

the rate of oxygen consumption when compared to the values obtained with animals receiving the control chow and high fat diets. Tetraiodothyroacetic acid (Tetrac) and Triiodothyropropionic acid (T_3 PA) also produced a slight elevation in oxygen consumption and reduction in growth rates although these values were not different statistically from those obtained with control animals (Table 2). Therefore the differences may be due to random variation. All of the remaining derivatives employed in experiment 1 (Table 2) were quite effective in reducing plasma cholesterol levels

TABLE 2. METABOLIC EFFECTS OF VARIOUS THYROXINE DERIVATIVES ADMINISTERED FOR TWO WEEKS AT A DOSAGE OF 0.5 MICROMOLE PER 100 GM. BODY WEIGHT PER DAY

Regimen	Ave. wt. gain†	Ave. O_2 consump.†	Plasma chol. concen. (mg. %)	Ave. liver chol. concen. (gm. %)	Ave. liver lipid concen. (gm. %)
Purina Chow	100	100	77	0.30	9.10
HFD (16% casein)	73	100	438	6.35	27.40
HFD +Thyronine	80	79	243*	6.15	26.73
HFD +3,5 Diiodothyronine (T_2)	50	92	190*	5.89	25.94
HFD +3,5,3′ Triiodothyronine (T_3)‡	24*	155*	251*	5.69	23.06
HFD +3,5,3′,5′ Tetraiodothyronine (T_4)	40*	137*	221*	5.92	23.44
HFD +3,5 Diiodothyroacetic acid (Diac)	81	95	226*	4.80*	21.88*
HFD +3,5,3′ Triiodothyroacetic acid (Triac)	39*	153*	187*	4.82*	19.71*
HFD +3,5,3′,5′ Tetraiodothyroacetic acid (Tetrac)§	63	114	202*	5.74	24.37
HFD +3,5,3′ Triiodothyropropionic acid (T_3-PA)	61	114	192*	5.93	22.44
HFD +3,5,3′,5′ Tetraiodothyropropionic acid (T_4-PA)§	100	101	251*	5.35	23.96

* Statistically significant difference from HFD value ($p < .01$).
† Expressed as % of chow diet values (70.0 gm. wt. gain/2 weeks and 6.0 liters of O_2 consumed/M^2/Hr.).
‡ The concentration of T_3 injected was reduced to 0.1 micromole/100 gm. body wt./day at the end of the first week of injection, because of the death of two animals from T_3 toxicity.
§ Since these experiments were performed, Stasilli *et al.* (19) have shown that Tetrac and T_4-PA undergo decomposition in alkaline solution. Therefore, the actual amount of active compound injected is unknown.

although diiodothyroacetic acid (Diac) was the only one that also significantly reduced liver cholesterol and lipid concentrations. There may have been some loss of Tetrac and T_4 PA activity since these substances were dissolved in dilute alkali (19). All of the other derivatives probably remained stable during the two-week period of injection.

In the second experiment, other available thyroid analogues were assayed under the same experimental conditions except that the protein level in the diet was elevated from 16% to 19% casein at the expense of the carbohydrate. Although a suboptimal dietary intake of protein may produce a more severe condition of hypercholesterolemia (15), it was decided to eliminate as many variables as possible and to make the diet more nutritionally balanced by elevating the protein level. The data obtained in this experiment are summarized in Table 3. The derivative triiodothyroethylamine (T_3 ethylamine) can be eliminated from further consideration since it produced an elevation in oxygen consumption and a reduction in growth. Triiodothyroformic acid (T_3 FA) and tetraiodothyroformic acid (T_4 FA) may also be eliminated since they failed to produce any significant effect on tissue cholesterol and lipid concentrations. Diiodothyroformic acid (T_2 FA) showed a preferential effect on tissue cholesterol values without

TABLE 3. METABOLIC EFFECTS OF VARIOUS THYROXINE DERIVATIVES ADMINISTERED FOR TWO WEEKS AT A DOSAGE OF 0.5 MICROMOLE PER 100 GM. BODY WEIGHT PER DAY

	Ave. wt. gain†	Ave. O_2 consump.†	Ave. plasma chol. concen. (mg. %)	Ave. liver chol. concen. (gm. %)	Ave. liver lipid concen. (gm. %)
Chow	100	100	66	0.24	10.80
HFD (19% casein)	100	100	252	6.04	23.24
HFD +3,5,3′5′ Tetraiodothyronine (T_4)	78*	133*	219*	5.65	16.94*
HFD +3,5,3′ Triiodothyroethylamine (T_3 ethylamine)	74*	140*	265	5.54*	19.41
HFD +3,5 Diiodothyroformic acid (T_2-FA)	104	115	204*	4.86*	21.00
HFD +3,5,3′ Triiodothyroformic acid (T_3-FA)	110	92	257	5.59	21.40
HFD +3,5,3′5′ Tetraiodothyroformic acid (T_4-FA)	106	111	256	5.99	25.66
HFD +3,5 Diiodothyropropionic acid (T_2-PA)	98	96	232	5.14*	20.96

* Statistically significant difference from HFD value ($p < .01$)
† Expressed as % of chow diet values (60.8 gm. wt. gain/2 weeks and 6.8 liters of O_2 consumed/M^2/Hr.).

significantly altering the growth rate and oxygen consumption of these animals, and diiodothyropropionic acid (T_2 PA) may also deserve further consideration because of its effect on liver cholesterol concentrations.

DISCUSSION

"Normal" plasma and liver cholesterol concentrations in the rat average 50–80 mg. %, and 0.200–0.400 gm. % respectively (9, 16). When the hypercholesterolemic diet containing cholic acid was fed, plasma and liver cholesterol concentrations were elevated from the control chow diet values of 77 mg. % and 0.300 gm. % to 438 mg. % and 6.350 gm. %, respectively. Although cholesterol feeding alone will produce some elevation in plasma and liver cholesterol concentrations, cholic acid was probably responsible for the large increases observed. It is believed that cholic acid markedly reduces the mobilization of liver and serum cholesterol, while showing little effect on cholesterol absorption (17, 18). A low protein diet will also elevate serum cholesterol concentration (15) and the 16% casein diet employed in the first experiment produced plasma cholesterol values of 438 mg. % as compared with 252 mg. % obtained with the 19% casein diet in the second experiment. However, both diets gave essentially the same liver cholesterol levels. These observations coincide with those presented by Moyer *et al.* (15).

Daily doses of 1–6 mg. of Triac and Tetrac have been reported as producing a temporary reduction in plasma cholesterol values in human subjects with myxedema or hypercholesterolemia (8–12). Under our experimental conditions, Triac was active in suppressing plasma and liver cholesterol levels (Table 2), but both Triac and Tetrac also produced some effect on oxygen consumption and growth. If larger amounts of these materials are required to control tissue cholesterol concentrations clinically, their usefulness might be questioned because of their effect on basal metabolism (Table 2 and reference 19).

Triiodothyropropionic acid (T_3 PA) has also been suggested as a thyroid

derivative for use in controlling cholesterol metabolism (10), but its calorigenic activity may also interfere with its usefulness (11, 19). In our experiments (Table 2), T_3 PA produced a significant reduction in plasma cholesterol but was without effect on liver cholesterol and lipid concentrations. There was also a statistically insignificant increase in oxygen consumption.

Duncan and Best (9) found that T_4 FA was more effective than thyroxine in lowering tissue cholesterol levels when both substances were administered in comparable goiter preventing doses. Subcutaneous injections of T_4 FA at a concentration of 5.44 micromoles per 100 gm. body weight per day reduced serum and liver cholesterol concentrations from 153 mg. % and 1.126 gm. % to 100 mg. % and 0.268 gm. %, respectively on a goitrogenic diet containing thiouracil, cholesterol, and cholic acid. On our experimental diet devoid of thiouracil, T_4 FA at a concentration of 0.5 micromole per 100 gm. body weight per day was without effect on plasma and liver cholesterol concentrations, whereas a compound such as diac produced a marked reduction in these values.

Therefore, of the thyroxine derivatives tested under the present experimental conditions, Diac and T_2 FA show the greatest preferential effect on plasma and liver cholesterol concentrations without significantly influencing oxygen consumption and growth. The other diiodo-derivatives T_2 and T_2 PA were considerably less effective in lowering cholesterol concentrations. It is possible that the more calorigenically active substances such as triiodothyronine and thyroxine might have produced the same degree of reduction in plasma and liver cholesterol concentrations at a markedly lower dose. Therefore, in order to determine whether a true preferential effect on tissue cholesterol levels is attributable to Diac, it is planned to compare the calorigenic, growth, and cholesterol concentration responses of Diac and thyroxine at different dose levels. It should also be noted that none of the thyroxine derivatives lowered the plasma and liver cholesterol concentrations to normal levels. The reasons for this may have been that the dosage was suboptimal or that the period of administration was of insufficient duration. There is also the distinct possibility that "normal" tissue cholesterol levels cannot be obtained with larger amounts of the derivatives because of the experimental conditions employed to produce hypercholesterolemia.

Although the oxygen consumption rates obtained in these experiments are based on a single dosage level, they are in substantial agreement with those obtained by Stasilli *et al.* (19) from dose response curves. These investigators found that 1) Triac was more potent than Tetrac, whereas Diac had less than 3% of L-T_4 activity, 2) T_3 PA was more active than T_4 PA, and T_2 PA had less than 3% of L-T_4 activity, 3) the propionic acid derivatives as a group were less active than the acetic acid derivatives, and 4) the formic acid analogues were less than 3% as active as L-T_4. Only in the latter case do our data differ slightly from those of Stasilli *et al.* In our ex-

periments, T_2 FA and T_4 FA produced oxygen consumption rates of 115 and 111% of control values respectively (Table 3), although neither increase was statistically significant from the control group values.

From the data obtained, it would appear that the maximum molecular requirements for controlling tissue cholesterol concentrations without significant calorigenic stimulation include a diphenyl ether structure containing iodines in positions 3 and 5, a carboxylic acid side chain no longer than 1 or 2 carbons and a phenolic group in the para position of ring B. It is possible that the iodines may be unnecessary since thyronine appears to have some effect in reducing plasma cholesterol values.

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RESERPINE AND THE CHRONIC STRESS OF REDUCED BAROMETRIC PRESSURE IN FEMALE RATS. OBSERVATIONS ON BODY WEIGHT, ADRENAL WEIGHT AND HISTOLOGY AND HEMATOCRIT VALUES[1]

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ABSTRACT

Sprague-Dawley rats were given reserpine in two dose patterns at ground level and at several patterns of low barometric pressure exposure. The latter was at 18,000 and 27,000 feet altitude equivalent levels. Observations were made on body weight, adrenal weight, histology and hematocrit levels.

Rats receiving reserpine lost weight, both at ground level and at altitude. Rats at ground level given an initial heavy dose of reserpine lost much weight but regained weight subsequently even though they were injected daily with small doses. Rats on small doses throughout lost weight at altitude, but placebo-injected and reserpine-injected rats lost weight as well.

Adrenal glands decreased slightly in absolute weight in placebo treated animals at altitude. Rats receiving an initial heavy dose of reserpine showed an increase in absolute adrenal weight, and an increase relative to body weight. The small dose of reserpine yielded a decrease in absolute weight but a smaller loss of weight relative to the terminal body weight. There was loss of fat in the adrenal gland with reserpine, especially at altitude, and vascular engorgement was seen at the higher altitudes when reserpine was given. Hematocrit levels increased in all animals at altitude, but more so in rats receiving reserpine.

Reserpine, in the dosage and patterns administered, did not prevent or reduce acclimatization changes as judged by loss of body weight, change in adrenal size, adrenal histology or blood hematocrit levels. On the contrary, the drug served as an added stress upon the organism in these respects.

THERE has been much interest recently in the effect of tranquillizing drugs on the response of the body to stress (1). The stresses that are applied are usually acute in character, such as cold, anesthesia, drugs (2). Little work has been done on the effect of tranquillizing drugs under stressful conditions of long duration, such as may be achieved by putting an animal under reduced barometric pressure for a period of one to two weeks. Acclimatization involves an acute phase of a homeostatic nature and

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adaptive changes of an endocrine and metabolic nature subsequently. Acclimatization as a stress was imposed on rats in the present study.

Of the several tranquillizing agents available, reserpine[3] was used because of its demonstrated effect regarding the blocking of the neurosecretory effects of the hypothalamus on the hypophysis with respect to ACTH (1, 3) and to gonadotrophic hormone (4). Female rats were employed, because female rats have been found to withstand better than males the effects of reduced barometric pressure (5).

METHODS AND MATERIALS

One hundred and sixteen albino Sprague-Dawley rats in good health were used. At the outset they were between 180 and 195 gm., and so were mature. They were housed under comparable conditions of lighting, temperature and ventilation, two to a cage. During the control period, the cages were kept in two low pressure chambers at baro-

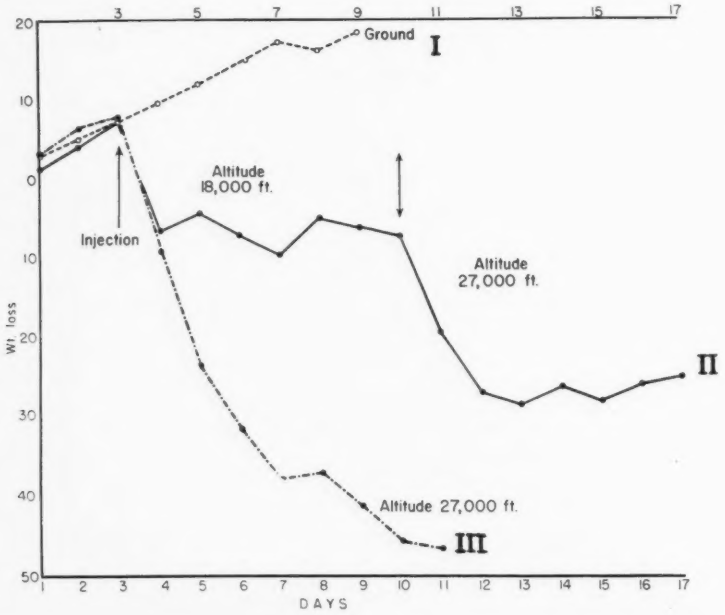

FIG. 1. Comparison of weights of rats at ground level (curve I), at 27,000 feet (curve III) and in two steps, 18,000 feet and then to 27,000 (curve II). Note that rats at ground level gain weight, rats taken to 18,000 feet for one week lose 10–12 grams in weight but are, through acclimatization, protected against severe weight loss when taken to 27,000 feet. The further weight loss is a little over 20 gm., for a total weight loss of 30 gm. In contrast, rats taken in one step to 27,000 feet lose weight progressively but at a decreasing rate for a week. The net weight loss is 50 gm., the virtual weight loss about 60 gm.

[3] Supplied by Ciba.

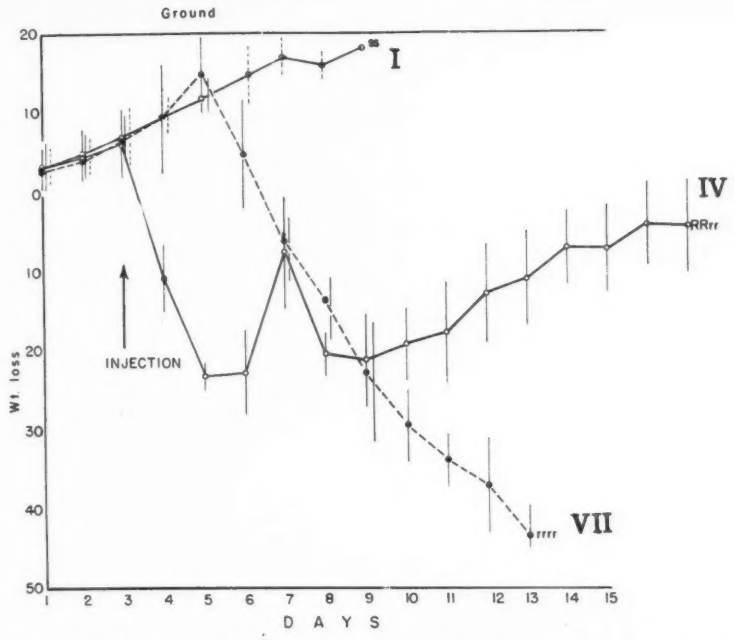

FIG. 2. Comparison of weights of rats at ground level given saline (curve I, ssss) small daily doses of reserpine (curve VII, rrrr) and two large initial daily doses followed by small doses of reserpine (curve IV, RRrr). Note that saline-injected rats gain weight, the RRrr rats lose weight (30 gm.), and then slowly regain weight for a net loss of 10 gm. while those on the daily low dose of reserpine (rrrr) continued for two days to gain weight, then lost progressively a total of 55 gm. in the following week.

metric pressure, ample ventilation being provided. During this time good eating and drinking conditions were maintained. At the end of the control period certain rats were taken for various determinations. These included blood hematocrit level, and adrenal weights and histology. Only those rats whose gain in weight was consistent with the average were chosen for further study. The selection of nine groups was on a random basis. The controls were as follows:

A. *Controls* (saline injected)

I. Kept at ground level throughout and injected daily with physiological saline in volume equal to the volume in the dose used in the experimental animals. (curve I, Figs. 1 and 2).

II. Kept seven days at an altitude equivalent to 18,000 feet ($\frac{1}{2}$ atmosphere), then raised to 27,000 feet, or altitude equivalent to one-third of an atmosphere for one week, and similarly injected with saline (curve II, Fig. 1).

III. Kept one day at 18,000 feet, then seven days at an altitude of 27,000 feet and similarly injected with physiological saline (curve III, Figs. 1 and 3).

B. *Experimental* (*reserpine-injected*)

IV. Kept at ground level, given two successive daily doses of 0.5 mg. reserpine per 100 gm. of body weight, two days abeyance, then subsequent daily doses of

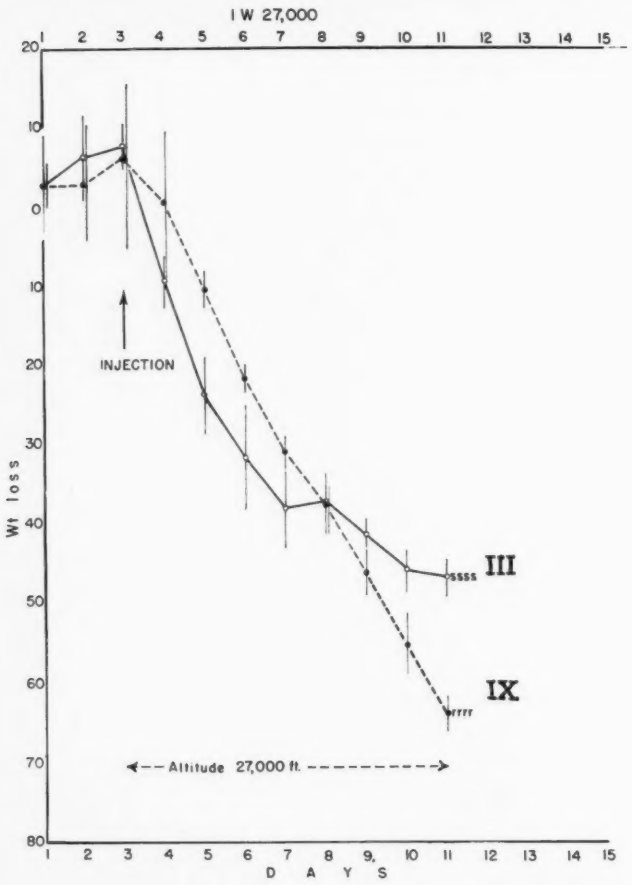

FIG. 3. Comparison of weights of rats at 27,000 feet given saline injections daily (curve III, ssss) and a low daily dose of reserpine (curve IX, rrrr). Note that the latter lose less weight for the first four days (35 gm. as compared with 45 gm.), although the saline-only rats fared better later (−50 gm. versus −70 gm.).

0.05 mg. reserpine per 100 gm. of body weight (RRrr, curve IV, Fig. 2).

V. Same as group IV, but adapted at 18,000 feet barometric pressure equivalent for one week then increased to 27,000 feet altitude equivalent for eight days (not illustrated; see Tables 1, 2 and 3).

VI. Same as IV and V but taken to 18,000 feet for one day and then kept at an altitude equivalent to 27,000 feet for one week (not illustrated; see Tables 1, 2 and 3).

VII. Kept at ground level for thirteen days and given 0.05 mg. reserpine per 100 gm. of body weight daily (rrrr, curve VII, Fig. 2).

VIII. After seven days at 18,000 feet barometric pressure equivalent, *then* received reserpine as group VII and raised to 27,000 feet altitude equivalent (a pre-adapted group) (not illustrated; see Tables 1, 2 and 3).

IX. At 27,000 feet barometric pressure equivalent for seven days received 0.05 mg. of reserpine in 100 gm. body weight daily (rrrr, Curve IX, Fig. 3).

Body weights were recorded daily and the animals supplied with food and water *ad libidum*. Purina dog chow was the food. Cages were cleaned twice weekly. The low pressure chambers were well ventilated and lighted at all times. All injections were given subcutaneously.

At the end of one week there was no mortality in any of the groups. A few rats were selected at random from each group for sacrifice. Those at altitude, after compression to ground level, were weighed, then killed by instant beheading. Blood samples were heparinized for hematocrit determinations. Adrenal glands were dissected out as quickly as possible, weighed, and one of each pair preserved in ten per cent neutral formol in physiological saline and stained with hematoxylin and eosin. The other gland of each pair was frozen-sectioned, stained with Scharlach stain.

At the end of the first week, rats in groups II, V and VIII were subjected to more severe hypoxia by lowering the barometric pressure to an altitude equivalent to 27,000 feet. Groups III and VI were no longer continued. At the end of two weeks, all remaining animals were sacrificed for study.

RESULTS

The results are summarized in Tables 1, 2 and 3, and in Figures 1, 2 and 3. The placebo (ssss) was physiological saline injected in a volume equal to that of the reserpine used. The symbols RRrr refer to two initial days of heavy reserpine administration followed by two days of no injection after which a small daily dose of reserpine was resumed. The symbols rrrr refer to the fact that the small dose of reserpine was used throughout. The Roman numerals refer to the groups of rats described under materials and methods, above.

Body weight (See Figs. 1, 2 and 3)

Rats at ground level gained weight throughout the period of observation. At 18,000 feet, they lost weight for one day, then held their weight until the pressure was reduced to a level equivalent to that at 27,000 feet, when they lost more weight. Rats taken to 27,000 feet after one day at 18,000 feet lost much more weight than the previous group.

Reserpine given to rats at ground level had, in a sense, a similar effect to altitude according to the pattern of administration. Thus a large initial dose of reserpine with two days of respite followed by smaller doses of reserpine caused no further loss of weight. In fact, the rats regained some of the lost weight. In contrast, rats put on the smaller dose of reserpine lost weight steadily for a week. Rats given the smaller dose of reserpine while at 27,000 feet altitude equivalent lost weight at the same rate, but not at first as rapidly as rats injected with a placebo alone. Later, these reserpine-treated rats continued to lose weight whenever the placebo-treated rats lost less weight. It appears that the low dose of reserpine protected a little the rats against the impact of severe continued stress but only for a limited time.

TABLE 1. ADRENAL WEIGHTS (MG.) IN CONTROL AND EXPERIMENTAL RATS
(Parentheses, adrenal weights per 100 gms. body weight, n, number of animals)

	Placebo	RRrr	rrrr
Ground 13 days	n = 13 54.8 (28.0) $\sigma = 5.4$	n = 11 50.0 (31.6) $\sigma = 5.4$	n = 12 51.3 (38.9) $\sigma = 5.9$
18,000 1 week	n = 4 51.2 (29.1) $\sigma = 3.1$	n = 6 56.2 (39.9) $\sigma = 5.0$	n = 0
27,000 ft. 1 week	n = 8 47.0 (30.5) $\sigma = 5.2$	n = 0	n = 5 51.1 (40.4) $\sigma = 5.7$
2 weeks	n = 9 41.3 (26.9) $\sigma = 1.8$	n = 12 57.2 (55.7) $\sigma = 5.3$	n = 11 49.9 (44.8) $\sigma = 5.2$

Adrenal weight (see Table 1)

Adrenal weights showed no gross or relative hypertrophy in normal rats under moderate or severe chronic altitude stress. Rats on the lower dose of reserpine showed no gross hypertrophy but did exhibit an increase in size relative to the decrease in body weight that took place. This was an established effect at ground level, but increased at high altitude. With two large initial doses of reserpine, followed by smaller daily doses, the gross and relative hypertrophy were even more marked, except at ground level.

Adrenal histology (see Table 2)

Adrenal histology showed with severe altitude stress a tendency to decreased fat, especially in the fasciculata and a tendency toward vascular engorgement. With reserpine, these effects are more marked, occurring to a degree even at ground level, but becoming increasingly marked at altitude. Some variation exists with the pattern of altitude stress and reserpine dosage.

Hematocrits

Only a limited number of hematocrit determinations were performed. These are summarized in Table 3. A more complete study of the red blood cell counts, hemoglobin contents, reticulocyte and eosinophile counts was not made, as in the study by Magill and Marbarger (5). Even so, some limited information is gained by examination of Table 3.

It is clear that the hematocrit level at 27,000 feet both in the controls and the reserpine-treated groups at ground level exceeds that at 18,000 feet. This is a result of acclimatization to low barometric pressure. Be-

TABLE 2. COMPARATIVE HISTOLOGICAL FEATURES OF CONTROL AND STRESSED ADRENAL GLANDS

	Controls ssss	RRrr	rrrr
14 days at ground level	(1a) All zones rich in fat, no vascular engorgement, sudanophilic zone	(1b) Glomerulosa free of fat; much fat in reticularis and fasciculata; no sudanophilic zone. Except for latter, gland looks normal, no vascular engorgement	(1c) Slight increase of fat in reticularis; otherwise normal
7 days at 18,000 ft.	(2a) Inner fasciculata has no fat; outer fasciculata has good fat deposit; reticularis has more fat than (4a); sudanophilic zone stronger than (4a)	(2b) Looks rather normal in all zones except that fat is more finely dispersed. Hyperplasia of the reticularis.	(2c) No sections made
7 days 18,000 ft. then 3 days at 27,000	(3a) Lower fasciculata and reticularis have engorged blood vessels and are full of fat; fat in glomerulosa is in coarse deposits as in (4a)	(3b) Completely free of fat in all zones. Vascular engorgement in reticularis and fasciculata. Rather similar to (4a)	(3c) Remarkable loss of fat in all zones; only a little fat in glomerulosa. Blood vessels engorged all way to the glomerulosa
1 day at 18,000 ft. then 7 days at 27,000	(4a) Lower fasciculata has no fat; outer fasciculata has much fat; reticularis has little or no fat. No blood vessel engorgement		(4c) Fairly uniform fat, dispersed but less than in control (4a); reticularis has engorged blood vessels and punctate distribution of fat; less fat in sudanophilic zone, although perhaps an overall increase in fat per section

tween the control group at 18,000 feet and the RRrr-treated group there is no difference indicated. Between the controls and the reserpine-treated group at 27,000 feet for two weeks, however, there is a greater concentration of erythrocytes than in the placebo-treated controls. There was no interference with, or abatement of, acclimatization, therefore, in the reserpine-treated animals of either group; in fact, the response of acclimatization to altitude, judged by this index, was increased substantially if no other contravening factors were involved. It may be noted that the reserpine-treated rats had watery feces, although water was supplied *ad libidum*.

DISCUSSION

The results reported above have a number of points in common with the work of Magill and Marbarger (5) and others (6, 7), on one hand, and with that of Wells, Briggs and Munson (2) and Darrow and Sarason (7) on the other.

TABLE 3. HEMATOCRIT VALUES OF CONTROL AND EXPERIMENTAL ANIMALS

	Placebo	RRrr	rrrr
Ground 13 days	n = 0	59% n = 6	49% n = 2
18,000 ft. 1 week	68% n = 3	70% n = 2	n = 0
27,000 ft. 1 week	n = 0	n = 0	79% n = 5
2 weeks	75% n = 8	82% n = 12	85% n = 11

The loss of body weight relative to adrenal hypertrophy and increased hematocrit levels at altitude, especially the higher altitude, employed, are typical of those reported before (5, 6). They need not be elaborated upon again. With respect to reserpine, it is evident that the large initial dose caused but slight adrenal hypertrophy and no absolute enlargement. However, it did confer, presumably through an initial alarm action, protection against the smaller doses which followed. This is evident in the body weight loss curve as compared with that which is found when only the smaller dose is used (see Fig. 2). Moreover, the latter group did show a relative, but not absolute, adrenal enlargement, resulting from the greater loss of body weight. Wells *et al.* (2) found that a single large dose of reserpine caused acute secretion of ACTH by the hypophysis which resulted in decreased ascorbic acid in the adrenal four hours later. Daily administration of reserpine thereafter had no such effect, for chronic administration of 0.125 mg. of reserpine per 100 gm. of body weight thereafter elicited no further secretion of ACTH. This observation, if it applies to the conditions of our experiments, would account for the normal acclimiztiation which occurred in our rats at altitude. The fact that the acclimatization response was exaggerated in the reserpine-treated rats as compared to the controls suggests that the treated rats were not deprived by the large doses of reserpine of the normal acclimatization mechanism and that the greater adrenal hypertrophy that occurred was a response of the endocrine system to the greater stress of the drug under chronic low barometric pressure. Wells *et al.* (2) observed, as did we, the decline of body weight with reserpine. The absolute weights of the adrenal glands of their treated rats, like our control rats, showed no increase in size.

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STUDIES ON ULTRACENTRIFUGE FRACTIONS OF ANTERIOR PITUITARY WITH SPECIAL REFERENCE TO ADRENOCORTICOTROPHIC HORMONE[1]

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ABSTRACT

Subcellular fractions from the anterior pituitary lobe obtained by ultracentrifugation were analyzed for purity by chemical, enzymatic, and physical tests. After separation of particles was established each fraction was analyzed by a three-dose response method for the hormones present. The hormones of the anterior pituitary occur in separate subcellular fragments; LH and STH in the secretory granules, TSH in the mitochondria, and ACTH in the microsomes and supernatant. FSH is scattered over several fractions. Treatment of the fractions with acid has little effect on STH while alkali destroys this hormone. TSH activity is destroyed by both acid and alkaline treatment. Acid treatment of the fractions augments FSH activity while it destroys LH activity. Alkaline treatment decreases the activity of the gonadotrophins. ACTH activity is potentiated by acid treatment, while alkaline treatment decreases the activity of ACTH slightly. Analysis of the fractions for hormone content indicates that ACTH comprises 1%, STH 0.6%, TSH 1.8%, FSH 1.1% and LH 1.4% of the weight of whole fresh anterior pituitary. On the basis of unitage there were 11 units of ACTH, 6 units STH, 720 units of TSH, 550 units of FSH and 350 units of LH per gm. of fresh pituitary and 6 times these amounts per gram of pituitary proteins.

IT IS well known that treatment of protein hormones with acid or alkali alters the potency of the preparation and may also alter the biological character of the hormone. For example, C. H. Li (13) demonstrated that an increase in ACTH activity could be obtained by partial hydrolysis of the whole protein molecule. Li and Pedersen (17) also observed that a dissociation of the protein molecule occurred in acetic acid solution. The authors suggest the possible existence of *more than one active center* on the protein hormone. Stack-Dunne and Young (25) also considered the possibility that *more than one ACTH* existed as they were able to separate an adrenal weight gain factor (AWG) from an ascorbic acid depleting (AAD)

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factor. Similar results were obtained by Rinfret (22). Lock (18) found the AAD factor of ACTH from pig glands to be completely destroyed by heating for ten minutes at 100° C with 0.1N NaOH while Hungerford *et al.* (9) and Johnsson (10) found the eosinophil reducing activity remained unchanged upon alkaline treatment again suggesting a dual activity of the hormone.

Bates (1) made a comparison of the effect of acid or alkaline extracts of the same preparation of ACTH upon the AAD activity. He found that the acid extracted fraction was more potent than the alkaline treated fraction in depleting the adrenal ascorbic acid when injected I.V. However, the reverse was true when the fractions were injected subcutaneously. The fractions were about equal in potency as assayed by the adrenal weight increase method. Dasgupta and Young (4) observed that their crude extracts of ox ACTH were devoid of activity in the Sayers test but activity rapidly appeared in the extract when it was adjusted to pH 3 with HCl. In every case the activity was in the supernatant and not in a precipitate resulting from treatment. Activity in the Sayers test was destroyed within two hours upon standing at alkaline pH. Experiments by Slusher (24) suggest a separation between the adrenal ascorbic acid depleting and corticosterone producing factors in experiments on rats subjected to stress after bilateral electrolytic lesions were placed in the hypothalamus. All of this evidence suggests the production of two types of ACTH.

With regard to other hormones, Collip (3) found that pituitary gonadotrophins and thyrotrophin were inactivated by prolonged boiling and were sensitive to alkali. Several workers have investigated the activity of growth hormone (STH) preparations at different pH's. Reid failed to observe a change in activity of STH after incubation of the hormone at pH 2.8 for 100 min. at 37° C. Ellis *et al.* (6) came to a similar conclusion after treating the hormone in acetic acid or acetate buffer. Li and Papkoff (16) found the activity to be destroyed by heating the hormone for one minute at 100° C in 0.1N acetic acid. Many groups agree that strong alkali destroys the growth hormone activity. It is interesting to note, however, that the acid treated hormone separated into two electrophoretic components. The biological activity appeared to be associated with the slower component.

Many investigators have reported that the activity of the trophic hormones from the anterior pituitary can be altered by various treatments but their results were obtained with purified hormones. In the work reported here fresh pituitary fractions obtained by ultracentrifugation were used, and comparative results obtained by these very different procedures can be discussed only on a general basis.

The purpose of this paper is to investigate the activity of the trophic hormones in the subcellular particulate fractions obtained by ultracentrifu-

gation following treatment with acid or base. Some observations on hormone content of the pituitary have also been made.

METHODS[4,5]

The methods of obtaining subcellular particles in distinct fractions from pig anterior pituitary lobes by ultracentrifugation and determining the purity of the fractions (11) have been described previously. The hormone content and localization in untreated fractions have also been reported (28). In general the method involved the separation of a nuclear fraction, secretory granules, mitochondria, light mitochondria, microsomes, and supernatant from a homogenate of pig anterior pituitary lobes in 0.25 M sucrose followed by identification of each fraction by enzymatic, biochemical, and physical tests. Each individual fraction was assayed for STH, TSH, LH, FSH, and ACTH. STH was assayed by the growth response in hypophysectomized rats and by the thymus weight increase of immature rats; TSH by the I^{131} uptake; LH by the increase in seminal vesicle and prostate weight of hypophysectomized rats and by the uterine weight increas in immature female rats. FSH was assayed by the testes weight increase in hypophysectomized rats and by the ovarian weight increase in immature rats using the augmentation method of Steelman and Pohley (27). ACTH was assayed by the Saffran (23) *in vitro* adrenal stimulation method, the decrease in circulating eosinophils, and the depletion of adrenal ascorbic acid. Each assay was conducted at three dose levels in the appropriate test animals.

After obtaining the subcellular fractions a part of the volume was set aside untreated. A volume of each fraction was brought to pH 3 with 0.1N HCl and maintained in a water bath at 100° C for thirty minutes. Similarly another portion of the original volume of each fraction was brought to pH 9 with 0.1N NaOH and kept in a boiling water bath for ten minutes. The acid and alkaline treated fractions were neutralized before injections into test animals. The fractions were injected not later than ten minutes after the described treatment. It should be emphasized that only two pH treatments were utilized. The method adopted was that used by C. H. Li (14) in his studies on pure hormones of the pituitary. This was necessary because of the prohibitive number of rats required to assay each fraction for each hormone over a wide pH range.

RESULTS AND DISCUSSION

When fractions obtained upon ultracentrifugation of anterior pituitary are treated with acid or alkali, characteristic changes occur in the activity of the individual hormones (Table 1 and 2). In order to discuss the changes conveniently, the hormones will be considered separately:

1. *TSH.* TSH is found largely in the mitochondrial fraction (Fraction III, Table 1). Treatment of the anterior pituitary fractions with acid (pH 3 with 0.1N HCl) and boiling for thirty minutes almost completely destroys the TSH activity (Table 2). After such treatment some activity is still located in Fraction III, but is only about 15% of that found in the untreated fraction. Alkaline treatment of the fractions (pH 9 with 0.1N NaOH) with boiling for ten minutes completely destroys TSH activity.

[4] Pituitary Glands were obtained through the courtesy of Armour and Company and White Provision Company, Atlanta, Georgia.

[5] The assistance of Mrs. Atalante Petkas is gratefully acknowledged.

TABLE 1. DISTRIBUTION OF HORMONAL ACTIVITY AMONG PITUITARY FRACTIONS ON THE BASIS OF HORMONE POTENCY IN A FRACTION AS COMPARED TO FRACTION I

Fraction	TSH	STH		FSH		LH & FSH		FSH	LH	
	Thyroid I^{131} uptake	Weight gain	Thymus wt.	Ovarian wt.		Uterine wt.		Testes wt.	Sem. ves. wt.	
				Normal	Acid	Normal	Acid		Normal	Acid
Homogenate	1.22[1,2]	.76	1.38	3.75	8.44	1.21	2.44*	—	—	—
I. Nuceli & debris	1.00	1.00	1.00	1.00	1.78*	1.00	4.73*	1.00	1.00	.17
II. Acidophilic granules	1.54	2.46*	1.50*	6.50*	17.2*	3.80*	3.31*	4.00*	1.74*	.35
III. Mitochondria	3.75	.43	.95	1.63	1.52*	1.66	1.23	2.13*	.53	.11
IIIA. Light Mitochondria	1.12	.48	.87	1.43	1.79	.97	.87	—	—	—
IV. Microsomes	.87	.00	.14	9.95*	9.95*	.58	1.57*	.82	.39	1.03*
V. Supernatant	1.35	.17	.73	1.90*	1.90	.17	1.76*	1.25	.98	—

[1] When potency values for acid or alkaline treated fractions are uniformly decreased, they are not included in the Tables.
[2] The potencies are calculated according to the method of Emmens (7), using Fraction I (Nuclei & debris) as standard. For purposes of calculation, dose response curves are assumed to be parallel.
* Values are significant (P =0.05).

The rapid destruction of TSH is in agreement with results reported by Geschwind (4) who noted that TSH is destroyed with periodate, and by Chow (2) who observed that TSH was inactivated by boiling alkaline treatment.

2. *STH*. The growth hormone activity is located largely in Fraction II (secretory granules). When acid treated fractions were assayed for STH by the growth response in hypophysectomized rats, the activity as compared to normal decreased in Fraction I, IIIA, IV, and V, and remained unchanged in Fraction II (Table 1). The lower values obtained in Fraction IIIA, IV, and V may indicate that some augmenting substance is destroyed. For example, the destruction of TSH which usually augments STH would explain the loss of activity in Fraction III. Alkaline treatment of the fractions reduces the STH potency as compared to the untreated fractions.

3. *FSH* and *LH*. The gonadotrophins were assayed by several methods

TABLE 2. THE ASSAY OF ACTH IN SUBCELLULAR FRACTIONS OF PIG ANTERIOR PITUITARY BY THE ADRENAL ASCORBIC ACID DEPLETION METHOD AND BY DECREASE IN CIRCULATING EOSINOPHILS UPON INJECTION OF A GIVEN NORMAL FRACTION AND OF THE SAME FRACTION TREATED WITH ACID OR ALKALI

An average of 40 rats was used to determine each value. Results are averages of five centrifugations.

Fraction	Adrenal ascorbic acid depletion			Decrease in circulating eosinophils		
	Normal	Acid	Alkaline	Normal	Acid	Alkaline
Homogenate	.90[1]	1.47	.71	1.32	.33	1.33
I. Nuclei & debris	1.00	.78	.63	1.00	.17	.00
II. Acidophilic granules	.83	.83	.90	.84	.03	.71
III. Mitochondria	.76	1.05	.82	1.53*	1.41*	.41
IIIA. Light Mitochondria	.83	1.12	.81	.87	.94	.00
IV. Microsomes	1.48*	2.94*	1.06	1.05	2.89*	1.29
V. Supernatant	.84	.83	.64	.73	1.77*	1.06

[1] See Table 1 for explanation of potency calculations.
* Data are significant (P =0.05).

in order to obtain some degree of confidence in location among the various subcellular fractions. Assays for FSH by the ovarian weight method of Steelman and Pohley (27) revealed activity in Fraction II, IV and V. Treatment with acid resulted in an augmentation of the FSH response. Alkaline treatment of the fractions on the other hand, uniformly decreased the ovarian weight below that seen with the untreated fractions and no definite peak of activity occurred. As assayed by the testes weight method in hypophysectomized male rats, FSH activity again appeared in Fraction II. Acid or alkali treatment of the fraction decreased the response (Table 1). It is possible that the much more sensitive ovarian weight assay method did not reveal the degree of destruction of FSH shown in the assay by increase in testes weight.

The assay of gonadotrophin in normal fractions by the uterine weight increase method in hypophysectomized rats reveals activity in Fraction II. Acid treatment did not alter the activity but alkaline treatment greatly diminished the response. The same situation is seen in the assay for LH by the increase in seminal vesicle weight in hypophysectomized male rats. In both assays an interesting problem arises. Upon acid treatment, Fraction IV (microsomes) was found to have LH activity as measured by both tests indicating that gonadotrophin is released in this Fraction.[6] The results could be summarized by saying that evidence favors the presence of LH and FSH in the secretory granules, that both appear to be decreased in amount on acid treatment, and that both are decreased to a still greater extent by alkaline treatment. The acid treatment appears to release LH from the microsomal fraction and this may reflect another state of the hormone. This result could not be explained as augmentation without assuming that either FSH or LH was released from an inactive state to provide augmentation. The fact that the response occurs in the seminal vesicle weight test supports that idea that the hormone released is LH, while the failure of the response to occur in the FSH-specific ovarian weight test of Steelman and Pohley suggests that FSH is not involved. The assays have been repeated on immature male and female rats in the 3-dose 96 hour test with identical results.

4. *ACTH*. The localization of ACTH in the untreated fractions is not as clear cut as that of TSH or STH (Table 2). As determined by the adrenal ascorbic acid depletion test ACTH is present in Fraction IV; the eosinophil response test reveals widely scattered activity with greater response in Fraction III; and the Saffran (13) *in vitro* steroid production test discloses activity in Fraction V, but with some activity in Fraction IV. On acid

[6] In our experiments the basophilic granules dissolve and presumably become a part of the supernatant or the microsomal fractions. McShan and Meyer (personal communication) have reported that centrifugation of pituitary in 0.88 M sucrose preserves the basophilic granules and that some gonadotrophic activity is seen in this fraction.

treatment Fraction III lost activity as measured by the eosinophil response test but the activity of Fraction IV and V was increased over that of the untreated fraction as estimated by this test. Alkaline treatment destroys that activity present in all fractions except in IV and V. The results suggest that a specific ACTH may be released from its carrier protein or activated from "pre-hormone." This is further supported by the ascorbic acid depletion test where the largest drop in adrenal ascorbic acid was seen in Fraction IV. Acid treatment markedly increases the activity of this fraction, with no increase in the activity of other fractions. When ACTH is assayed by Saffran's *in vitro* method and the steroid production determined by the non-specific ultraviolet adsorption at 240 μ (E-240) there is an increased steroid production in adrenals treated with the acidified fractions (Table 3). The largest single response is produced by Fraction V; the greatest response in terms of percentage increase above the untreated fraction is produced by acidified Fraction IV. When the corticosterone-specific fluorescence test (Zenker, 29) is utilized, the marked increase in steroid production with acidified fractions did not occur. This may indicate that the fractions (ACTH) act by affecting the precursors in the synthesis of corticosterone, but do not act directly in the final step. Alkaline treatment decreases but does not destroy the ACTH activity in all pituitary fractions.

The marked increase in the activity of ACTH upon acid treatment of a given fraction may be due to several mechanisms. (1) ACTH may be present in the gland in a larger, relatively inactive molecule from which a moiety is released on acid treatment; (2) "active" sites of the molecule may be made "available" by acid treatment; or (3) it is possible to assume that acid treatment destroys some "inhibitor" and releases the ACTH. All these explanations have some basis in fact. Several workers have demonstrated that fractions of the ACTH molecule (m.w. 20,000) with molecular weights of about 2500 are extremely active (17, 19, 20). Li and others (17, 13, 14) have also suggested that ACTH can be potentiated by acid treatment, and Stack-Dunne and Young (25) have claimed to be able to separate two fractions of ACTH, one which caused adrenal weight gain, and another which caused ascorbic acid depletion.

Regardless of the mechanism of action, it is apparent that treatment of subcellular fractions of anterior pituitary cells with acid enhances the ACTH activity markedly. The fact that treatment enhances the potency of ACTH in Fraction IV by the eosinophil depletion test where little activity existed before strongly supports the suggestion that acid treatment in some way "activates" ACTH.

The results on ACTH reported here confirm the work of others on pure hormone. It must be stressed, however, that a completely different situation exists. In this case hormones are separated physically, not chemically, and potentiation with acid may simply reflect a release from a particulate rather than actual activation of a protein molecule.

TABLE 3. *In vitro* SYNTHESIS OF STEROID BY ADRENALS AFTER ADDITION OF SUBCELLULAR PARTICLES OBTAINED BY ULTRACENTRIFUGATION

The data are averages of five centrifugations with eight flasks per fraction.

Fraction	Test	μg. steroid/100 mg. adrenal/hour/mg. N		
		Normal	Acid	Alkaline
Homogenate	E-240	7.5±1.1*	9.9±1.2	6.4±0.3
	Fluor.	5.3±0.01	5.3±1.2	3.0±0.4
I	E-240	6.8±0.01	14.6±1.0	6.4±1.6
	Fluor.	6.7±0.3	6.3±0.0	1.8±0.3
II	E-240	6.1±2.0	12.8±0.6	4.2±0.7
	Fluor.	6.6±1.4	5.5±0.7	4.9±0.06
III	E-240	8.7±1.7	18.7±1.5	7.2±1.4
	Fluor.	7.4±1.2	9.8±1.8	4.0±0.2
IIIA	E-240	8.1±3.0	21.3±2.1	5.0±1.4
	Fluor.	7.7±1.3	7.0±1.0	2.6±0.1
IV	E-240	9.2±1.0	28.8±0.5	3.6±0.2
	Fluor.	6.5±0.8	6.8±2.0	3.0±0.2
V	E-240	36.1±2.0	43.3±2.2	25.1±1.5
	Fluor.	24.9±5.6	26.2±1.2	18.4±0.4
ACTH				
10 μg. protein	E-240	2.8±0.2		
	Fluor.	1.1±0.1		
30 μg. protein	E-240	4.3±0.7		
	Fluor.	2.4±0.6		

* Standard deviation.
Blanks subtracted from all values.

Calculation of the grams of hormone per kilogram of protein in anterior pituitary reveals several interesting observations (Table 4). For this calculation each hormone was assayed against a standard preparation as indicated in the results. Dose response curves were plotted and the potency determined as dose of standard as compared to dose of fraction required to produce a given response. As determined by both thymus weight increase and growth rate, STH is present in secretory granules and both methods assay almost identical amounts of hormone. TSH is also present in this fraction but much more is present in the mitochondrial fraction. Essentially equal quantities of ACTH are present in microsomal fractions as determined by any method of assay. The greater amount of hormone present in the supernatant as revealed by Saffran's *in vitro* method suggests that a soluble form of ACTH is present here which more easily penetrates the cells and stimulates steroid production. High levels of both FSH and LH are present in the secretory granule fraction. It is more than likely that such high values are unreliable due to the well-known augmentation which occurs between these two hormones. Only small quantities of hormones with the exception of TSH and ACTH are present in the supernatant.

TABLE 4. THE HORMONE CONTENT OF ULTRACENTRIFUGE FRACTIONS OF PIG ANTERIOR PITUITARY

Results are expressed as grams of hormone per kilogram of pituitary protein (nitrogen ×6.25). The values given are averages of six centrifugations with approximately 50 animals per dose per fraction per assay. Significant increases above the average hormone content ($p = 0.02$) are indicated by asterisks. Unitage is calculated on potencies of pure hormone standards as indicated below.

Fraction	ACTH				STH		TSH	FSH	LH	Gm./kg. of all protein hormone in given fraction
	Ascorbic acid depl.	Eosinophil decrease	Saffran's test E-240	Saffran's test Fluorescence	Thymus wt.	Growth	I^{131} uptake	Ovarian wt.	S.V. wt.	
I. Nuclear	71	110	17	13	50	60	61	5	71	283
II. Secretary granules	52	56	19	27	136*	166*	166*	170*	310*	851†
III. Mitochondria	46	200*	70	70	25	30	250*	10	21	379
IIIA. Light Mitochondria	52	87	45	50	20	20	71	147*	10	320
IV. Microsomes	110*	100	120*	100*	0	5	37	41	33	226
V. Supernatant	56	71	181*	245*	0	5	140*	18	80	458
Average amount of given hormone in all fractions gm hormone/kg. pituitary protein	64	104	75	84	37	45	121	65	88	403 / 410
Units of hormone/gm. whole fresh pituitary	11[1]				6[2]		720[3]	550[4]	350[5]	—
Hormones/% of fresh pituitary by weight	1.1				0.6		1.8	1.10	1.4	6.0%
% increase in activity on acid treatment	+200	+240	+188	−21	0	0	0	+157	−65	

† These values are unrealistic and reflect augmentation.

[1] ACTH-Units based on International Reference Standards (1 unit = 1 mg.).

[2] STH-Units based on International Reference Standards (1 unit = 1 mg.).

[3] TSH-Units based on TSH averaging 40 units/mg. maximum potency as compared to the International Reference Standards (1 unit = 1 mg.).

[4] FSH-Units based on FSH averaging 50 units/mg. maximum potency as compared to Armour 264-151x (1 unit/mg.).

[5] LH-Units based on LH averaging 2.5 units/mg. maximum potency as compared to Armour 227-80 (1 unit/mg.).

Averaging the total amount of each hormone present in the pituitary protein indicates that about 7% *of the protein* is ACTH, about 4% is STH, about 12% TSH, 7%FSH, and 9% LH. The sum of all hormones within a given fraction reveals that by far the largest amount of hormone is present in the secretory granule fraction where *851* gms. of hormone *per kilogram* of protein were obtained. The high value is undoubtedly due to the augmentation between FSH and LH with resulting high gonadotrophin titers.

Calculating the total units of hormone theoretically present in one gram of pituitary protein discloses that ACTH averages about 70 units/gm.; STH about 40 units/gm.; TSH about 4800 units/gm. FSH about 3500 units/gm. and LH about 2250 units/gm. The values mentioned are higher than those usually given in the literature because they are based on milligrams of hormone protein per milligram of total protein instead of the usual milligrams of hormone or units of hormone per gram of fresh weight of anterior pituitary. In our experience the protein comprises about 15% of the weight of the fresh gland before centrifuging. Recalculation on the basis of fresh weight decreases all values to about one-sixth.

Drs. A. E. Wilhelmi and Stanley Ellis[7] (personal communication) have prepared many pituitary hormones as reference standards and their values have been taken in the calculation of hormone content as presented here. The results obtained by our method of isolation as compared to the protein separation methods of Wilhelmi and of Ellis are in excellent agreement for STH but in poor agreement for other hormones as our results are much higher. This may reflect losses which occur in separation procedures which are not found in whole gland assay, or augmentations between the various hormones in fractions. It should be emphasized that the maximum degree of purity which has been obtained by the ultracentrifuge method is about 15%.

If the various fractions are treated with acid as described above changes in total hormone content occur (Table 4). In fractions which contain ACTH as measured by non-specific methods, the activity is increased by more than 200% upon acid treatment. In the non-specific ultraviolet absorption method for identification of steroids (E-240) an increase of 188% occurs while in the specific fluorescence method measuring corticosterone production, no increase in activity occurs on acid treatment. This may indicate that acid treatment releases a form of ACTH which stimulates formation of precursors of the final product.

Interestingly enough, total ACTH activity in the pituitary assays the same by ascorbic acid depletion or by Saffran's *in vitro* method. Much higher amounts of ACTH are found when the hormone is analyzed by the eosinophil response again demonstrating the relative non-specificity of this method.

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[7] Thanks are due to the National Institutes of Health, Endocrine Study Section, to Dr. A. E. Wilhelmi, and to Dr. Stanley Ellis, for the generous supplies of the standard hormone preparations.

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EFFECT OF THYROTROPIN ON THE RELEASE OF IODIDE FROM THE THYROID

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ABSTRACT

The concentrations of trichloroacetic acid (TCA) soluble and TCA-precipitable iodine in the arterial and thyroid venous plasma of the pentobarbital-anesthetized dog were measured serially before and after the systemic administration of thyrotropin. When the glandular iodine had been labelled by administration of I^{131} 2 to 6 days earlier, the injection of thyrotropin resulted in a prompt release not only of PBI^{131} but also of $iodide^{131}$ from the gland, and there was a loss from the gland of appreciable quantities of $iodide^{127}$ as well. The release of iodide which was detectable within 30 minutes after TSH injection, was enhanced by the administration of perchlorate. Since thyrotropin had little effect on the thyroidal accumulation of plasma iodide under these conditions, the inorganic iodide released from the gland was not iodide newly-entered from the plasma, but arose from an iodine-containing precursor within the gland. A prompt stimulatory effect of TSH upon an intraglandular deiodination process appears to be the likeliest explanation of these observations.

BY THE use of radioactive iodine, the stimulating effect of thyrotropin (TSH) upon the thyroid gland can be shown in at least three different ways—the "trapping" of plasma iodide, the organic binding of iodine and the secretion of thyroid hormone. Increased glandular secretion of hormonal iodine is one of the earliest detectable responses to the administration of thyrotropin. Thus, in man while an interval of 8 to 10 hours elapses between injection of TSH and detectable enhancement of thyroidal radioactive iodine accumulation (1), the systemic plasma concentration of labelled PBI rises within 90 minutes (2) and the labelled PBI concentration in thyroidal venous plasma of the dog (3) and rabbit (4) rises within 30 minutes after TSH injection. In the course of studies involving measurements of the plasma concentrations of labelled iodine in arterial and thyroid venous blood of the dog (5) the effects of TSH administration upon these values were investigated. The experiments showed that TSH not only stimulated hormonal iodine secretion, but also induced a prompt

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glandular release of inorganic iodide which resembled in its time course and magnitude the increased hormonal iodine secretion.

METHODS

Nine mongrel dogs weighing 30 to 60 pounds each were used, each serving as the subject of a single experiment. The animals were used within a few days after their arrival in the laboratory and their previous dietary history was unknown; in the laboratory they were fed Purina Chow. Each animal but one received an intravenous injection of 50 to 100 μc. of I^{131} (carrier-free, sodium iodide^{131} (Squibb)) 2 to 6 days before the experiment was performed in order to label the glandular iodine; the remaining dog received the tracer I^{131} 2 hours before the experiment. The experimental procedure was designed to provide a series of simultaneously drawn samples of arterial and of thyroid venous blood; the experimental procedure lasted 1 to 2 hours and observations were made before and after administration of thyrotropin into a systemic vein.

The general operative procedure has been outlined (5). The animals were anesthetized with intravenously injected pentobarbital, further doses of pentobarbital being administered as required to keep the dog in a state of deep anesthesia throughout the experimental period. The femoral artery and vein in the left groin were dissected free. The distal end of the femoral vein was ligated and proximate cannulation performed with a piece of polyvinyl tubing equipped with a three-way stopcock; the patency of the tubing was maintained throughout the experiment by a very slow infusion of 0.9% saline solution. This cannula provided a convenient means for the administration of test substances into the systemic circulation. Exposure and dissection of the thyroid gland were made by a longitudinal midline cervical incision. After retraction of the sternocleidomastoid and strap muscles, the venous pattern of the thyroid, which in the dog consists of 2 discrete lobes, was studied and the lobe with the least prominent veins was removed. In most cases the arterial supply to the hemi-thyroid was provided by the superior thyroid artery which was in close proximity to a small superior thyroid vein. At the inferior pole of the hemi-thyroid a single thyroid vein (sometimes bifid) was present which drained into the internal jugular. All small tributaries into the inferior thyroid vein from the lower lobe of the thyroid to the internal jugular vein were ligated. The internal jugular was then ligated and when the inferior thyroid vein was moderately distended a short section of thin polyvinyl tubing (B-D. #442-T, O.D. 0.965 mm.; I.D. 0.58 mm.) was inserted into the jugular vein and advanced cephalad toward the inferior thyroid vein. In some cases the final position of the cannula tip was in the inferior thyroid vein while in others the tip was in the internal jugular; in all cases, all visible tributaries to the internal jugular distal to its ligation were ligated and divided. Thyroid venous blood samples were obtained from the vein by very gentle traction on the piston of a 2 or 5 ml. syringe attached to the hub of a 20 gauge needle threading the proximal end of the plastic cannula. Consecutive 2 to 4 ml. blood samples were then obtained from the thyroid vein throughout the remainder of the experiment, the period of blood sampling lasting 1 or 2 hours; the time required to obtain each sample was recorded. Arterial blood samples were obtained intermittently from the femoral artery by needle aspiration; the arterial samples were obtained approximately at the mid-point of the collection period for the corresponding venous sample. Arterial and thyroid venous blood samples were collected in heparin-coated syringes (a separate syringe being used for each specimen) and were transferred to 12 ml. graduated centrifuge tubes containing a drop of heparin solution. At the conclusion of the experiment the remaining thyroid lobe was removed and placed in a vial for subsequent determination of weight and radioactivity.

The volume of each blood sample was measured, and after centrifugation the packed cell volume recorded. The plasma was then removed and aliquots (0.50 or 1.0 ml.) were placed in test tubes for determination of total plasma radioactivity in a well-crystal scintillation detector. The samples were then treated with 8 ml. 10% trichloroacetic acid (TCA), and the precipitate, after washing with TCA solution, was dissolved in 2 ml. 2N NaOH. When solution was complete, the TCA-precipitable I^{131} was measured. Since some plasma samples showed evidence of hemolysis, 2 or 3 drops of 2.5% thiourea solution were added to each plasma sample before TCA precipitation in order to minimize the spurious increases in TCA-precipitable iodine which may occur when hemoglobin-containing solutions are acidified (6). From the values of total plasma I^{131} and TCA precipitable I^{131} (PBI^{131}) the TCA-soluble I^{131} or "inorganic" I^{131} was obtained by difference. In a few cases TCA-soluble I^{131} was determined in an aliquot of the supernatant solution from the TCA precipitation, and the values agreed well with those derived by difference. An appropriately diluted aliquot of the I^{131} solution administered to each dog served as a standard, and plasma I^{131} concentrations were expressed as a percentage of the dose per liter of plasma. Stable plasma iodine determinations (PBI and total) were performed in some specimens by the chloric acid method (7, 8). Thyroidal uptake of I^{131} was determined by measuring the radioactivity of the excised glands under the same geometrical conditions as an aliquot of the tracer dose. Thyrotropin (Armour "Thytropar"), 5 to 10 U.S.P. units in 5 ml. saline, and potassium perchlorate, 5 ml. of a 2% aqueous solution, were given by intravenous injection via the femoral vein cannula; the time required for injection of each substance was less than 1 minute.

The 8 experiments in which sufficient time elapsed between I^{131} administration and operation to permit appreciable labelling of glandular iodine may be divided into 2 groups of 4 experiments.

Group I. TSH alone, or before perchlorate (Experiments 1–4). The effect of TSH upon arterial and thyroid venous I^{131} concentration was studied; observations were made for 25 to 78 minutes after TSH injection, and in 2 experiments (Experiments 1 and 4) perchlorate was subsequently given, and its effects upon plasma I^{131} concentration noted. In Experiment 4, in this group, TSH was given before the neck dissection was completed and before any blood samples were drawn in order to permit a longer period of action of TSH.

Group II. Perchlorate before TSH. The four animals in this series (animals 5–8) were pretreated with perchlorate, and plasma samples were obtained before and after TSH was injected (24 to 56 minutes after perchlorate).

RESULTS

Some of the results of the TSH experiments in groups I and II are summarized in Tables 1 and 2. In all experiments, the PBI^{131} concentration in thyroid venous plasma during the control period was greater than that in arterial plasma and generally the venous $iodide^{131}$ concentration was slightly less than the arterial. The administration of TSH in all cases but one (experiment 2) resulted in an increase in the concentration of $iodide^{131}$ in thyroidal venous plasma, so that this value exceeded the corresponding pre-TSH value or the simultaneous arterial value. The PBI^{131} concentration of thyroidal venous plasma increased as a consequence of TSH injection in every case, and the time when the increments of venous $iodide^{131}$ and PBI^{131} became apparent were similar, ranging (6 experiments) from 18 to 34 minutes. In general the curves relating time after TSH administra-

TABLE 1. GROUP I EXPERIMENTS. EFFECT OF TSH ON ARTERIAL AND THYROID VENOUS PLASMA I^{131} CONCENTRATION; NO PRE-TREATMENT WITH PERCHLORATE

Experiment No.	Time after I^{131} given (days)	Thyroid uptake I^{131} (%)		Plasma I^{131} (% dose/l)* Inorganic I^{131} Control	TSH	Perchlorate	PBI^{131} Control	TSH	Perchlorate	Mean thyroid venous blood flow (ml./min.)	Duration of periods (min.)‡ TSH	Perchlorate
1	5	31	A†	0.51	0.50	0.56	0.74	0.67	0.61	0.73	29	24
			V†	0.39	1.04	4.06	1.18	2.73	5.21			
			(V-A)†	−0.12	0.54	3.50	0.44	2.06	4.60			
2	2	13	A	1.82	1.64	—	0.15	0.12	—	0.64	78	—
			V	1.43	1.28	—	0.51	0.63	—			
			(V-A)	−0.39	−0.36		0.36	0.51				
3	5	66	A	0.39	.35	—	0.20	0.20	—	0.22	34	—
			V	0.35	.74	—	0.24	0.67	—			
			(V-A)	−0.04	0.39		0.04	0.47				
4	6	27	A	—	0.30	0.34	—	0.27	0.24	0.30	65	42
			V	—	1.48	2.61	—	3.55	2.90			
			(V-A)		1.18	2.27		3.28	2.66			

* Plasma I^{131} concentrations are those existing at the end of period (control, TSH, perchlorate).
† A = arterial plasma; V = thyroid venous plasma; V-A = thyroid venous-arterial plasma concentration difference.
‡ In experiments 1 and 2 TSH dosage was 10 U.S.P. units, in experiments 3 and 4, 5 U.S.P. units. 100 mg. potassium perchlorate given at the end of TSH period in experiments 1 and 4.

tion and changes in iodide^{131} and PBI^{131} tended to be similar (Fig. 1). In Experiments 1 and 3 of group I, 20 and 34 minutes respectively, elapsed between TSH injection and distinct increase in thyroid venous iodide^{131} concentration, and in experiments 5–8 of group II these values were 31, 33, 30 and 18 minutes. The experimental conditions were such that the time elapsing between TSH injection and distinct changes in plasma iodide^{131} and PBI^{131} concentrations is probably greater than the true value. Since

TABLE 2. GROUP II EXPERIMENTS. EFFECTS OF TSH ON ARTERIAL AND THYROID VENOUS PLASMA I^{131} CONCENTRATION; DOGS PRE-TREATED WITH PERCHLORATE

Expt. No.	Time after I^{131} given (days)	Thyroid uptake I^{131} (%)		Plasma I^{131} (% dose/L) Inorganic I^{131} Control	Perchlorate	TSH	PBI^{131} Control	Perchlorate	TSH	R_I*	R_{PBI}†	Mean thyroid venous blood flow (ml./min.)	Duration of periods (min.) Perchlorate	TSH
5	5	15	A	0.36	0.52	0.48	0.08	0.07	0.08			0.12	40	31
			V	0.36	0.59	0.73	0.25	0.16	0.19					
			(V-A)	0.0	0.07	0.25	0.17	0.09	0.11	3.6	1.2			
6	5	35	A	—	0.27	0.30	—	0.17	0.17			0.28	56	65
			V	—	0.40	1.17	—	0.23	0.52					
			(V-A)		0.13	0.87		0.06	0.35	6.7	5.8			
7	2	12	A	—	1.43	1.50	—	0.06	0.06			0.27	56	92
			V	—	1.75	3.16	—	0.20	0.60					
			(V-A)		0.32	1.66		0.14	0.54	5.2	3.9			
8	5	15	A	0.24	0.27	0.26	0.17	0.14	0.15			0.30	24	62
			V	0.20	0.33	3.50	0.21	0.21	3.16					
			(V-A)	−0.04	0.06	3.24	0.04	0.07	3.01	54	43			

* R_I = (V-A) for Iodide^{131} at end of TSH period/(V-A) at end of perchlorate period.
† R_{PBI} = (V-A) for PBI^{131} at end of TSH period/(V-A) at end of perchlorate period.
Potassium perchlorate dosage: 100 mg. in each experiment.
TSH dosage, 5 units in each experiment, except No. 5, 10 units.

the time noted included the entire time required to draw the particular sample showing the increment and since the time needed to obtain a single sample was sometimes 15 minutes or longer, the increment might have been detectable sooner had smaller and more frequent blood samples been taken. The latent periods for TSH-induced increments in PBI^{131} in thyroidal venous plasma agree well with those of Ackerman and Arons (3) obtained by a similar technique in the dog and are longer than those noted by Sodeberg in the rabbit (4). The effect of TSH on enhancing the venous concentration of $iodide^{131}$ persisted for at least 1 hour. This was shown in an experiment (Table 1, Expt. 4) in which the venous $iodide^{131}$ concentration

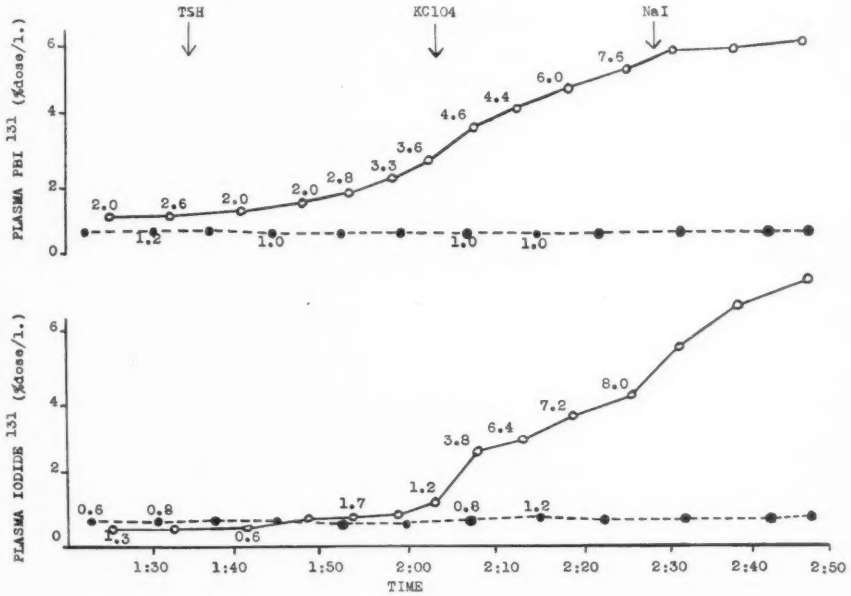

FIG. 1. Effect of thyrotropin on arterial and thyroid venous plasma concentration of inorganic and protein-bound iodine.

In this and the remaining figures, solid lines (open circles) represent thyroid venous plasma, broken lines (solid circles) indicate arterial plasma. The time of intravenous injection of designated substances is shown by arrows.

Arterial and thyroid venous plasma concentrations of PBI^{131} are shown in the upper panel and of inorganic I^{131} in the lower panel. The numerals about the points indicating I^{131} values are the values for the plasma concentrations (μg./100 ml.) of PBI^{127} (upper panel) and inorganic $iodide^{127}$ (lower panel) in the corresponding samples.

The animal (experiment 1) has been injected with a tracer dose of I^{131} 5 days before blood samples were obtained, the glandular uptake being 31%. Injection of TSH (10 U.) was promptly followed by increases in the concentration of TCA-precipitable and TCA-soluble I^{131} and I^{127} in thyroidal venous plasma. Administration of potassium perchlorate (100 mg.) and later of sodium iodide (1 gm.) led to further liberation of iodide into thyroid venous plasma.

was nearly 5 times greater than the arterial value 1 hour after TSH injection, under conditions where the pre-TSH venous iodide^{131}, although not measured, would not have been expected to exceed the arterial iodide^{131} concentrations; the effect persisted through the subsequent perchlorate period (42 minutes).

Of the 4 experiments in group I (TSH effects in animals not pretreated with perchlorate) an absolute increase in thyroidal venous iodide^{131} concentration was observed following TSH administration in 3 cases. In the remaining experiment (no. 2) the response was very small, despite the fact that thyroid venous PBI^{131} concentration was greater than the arterial value and displayed a slight increase after TSH, showing that the cannula was draining the thyroid venous outflow and that the gland was secreting organic iodine. The thyroidal venous iodide^{131} concentration actually fell from 1.43% to 1.28% dose/l. However, because the arterial iodide^{131} concentration fell during the TSH period, there was actually a very slight increment of venous iodide^{131} concentration after TSH in the sense that the arterio-venous iodide^{131} concentration difference became somewhat less negative.

An experiment in group I is shown in Figure 1 (Experiment 1) illustrating the effect of TSH in inducing a prompt increase in both venous inorganic and protein-bound I^{131} (the latter, in this case, being detectable somewhat earlier than the former); also shown are the generally parallel nature of the increase in PBI^{131} and iodide^{131} concentrations in blood leaving the gland and the effect of perchlorate on enhancing the increase in venous iodide^{131}, as shown by the increased slope of the venous iodide^{131} curve after perchlorate. Twenty-nine minutes after TSH injection, the thyroid venous-arterial plasma concentration difference for iodide^{131} had changed from a negative value indicating net glandular accumulation of iodide^{131} to a positive value indicating net loss of iodide^{131} from the gland, and within 24 minutes after perchlorate administration, this value had increased nearly 6-fold. The release of PBI^{131} from the gland was greatly enhanced after TSH administration, as shown by the 10-fold increase in the veno-arterial PBI^{131} concentration difference in the 53-minute period after TSH. The percentage increase in glandular release of iodide^{131} during the perchlorate period was nearly 3 times the corresponding value for PBI^{131}. The administration of a large quantity of sodium iodide evoked a further discharge of inorganic I^{131} from the gland, while the rate of PBI^{131} secretion failed to increase. Figure 1 also shows that stable iodine responses to TSH and perchlorate tended to parallel the I^{131} values. The relative constancy of the arterial labelled and stable inorganic iodide and PBI values shown in Figure 1 was a feature common to all the experiments.

The apparent enhancing effect of perchlorate on the TSH-induced thyroidal iodide discharge noted in experiment 1 was also noted in experiment

4, where perchlorate injection was followed by a nearly 2-fold increase in veno-arterial iodide difference, as compared with the value at the end of the period of TSH alone, although there was a slight decrease in the PBI^{131} secretion.

In the group II studies potassium perchlorate (100 mg.) was injected 24 to 56 minutes before giving TSH so as to inhibit the iodide concentrating mechanism of the gland. It was thought that if active glandular accumulation of circulating iodide were thus prevented, the release of $iodide^{131}$ from the gland induced by TSH not only might be more readily detected but also might be of greater magnitude than if the iodide concentrating mechanism were operating. In all 4 experiments (Table 2) the venous $iodide^{131}$ concentrations at the end of the perchlorate period were greater than the simultaneous arterial values; the venous-arterial $iodide^{131}$ concentration differences averaged 26% of the arterial values. The injection of thyrotropin then produced in each case prompt and progressive rises in the venous $iodide^{131}$ concentration, so that at the end of the TSH period (which ranged between 30 and 90 minutes) the veno-arterial concentration differences were 3 to 50 times greater than those at the beginning of the period. The increases in the concentration of PBI^{131} were of comparable magnitude; however, the thyroid veno-arterial concentration differences for PBI^{131} at the end of the TSH period as compared with those at the beginning were proportionally slightly less than the corresponding values for $iodide^{131}$ (columns R_I and R_{PBI}, Table 2). By contrast, in the experiments in group I (no perchlorate pre-treatment) the PBI^{131} response to TSH was proportionately greater than the $iodide^{131}$ response.

Figure 2 shows an experiment in group II (no. 8). It illustrates a difference between the perchlorate-induced increase in thyroid venous $iodide^{131}$ concentration and that following TSH injection. After perchlorate alone the venous $iodide^{131}$ concentration promptly rose to exceed the arterial value and remained above the arterial value in a plateau-like fashion or fell somewhat throughout the remainder of the perchlorate period. By contrast, TSH induced a further increase in venous $iodide^{131}$ concentrations which was not only of greater magnitude but increased progressively. The parallel time courses of the PBI^{131} and inorganic $iodide\text{-}I^{131}$ increments are also shown in Figure 2.

The possibility of an early effect of TSH upon thyroidal iodine accumulation was studied by measuring the arterio-venous $iodide\text{-}I^{131}$ concentration differences in a single dog injected with a tracer dose 2 hours before the first blood samples were drawn. In a 40-minute period after administration of TSH (10 U.S.P. units) no tendency for the large arterio-venous $iodide^{131}$ differences to increase was observed, and, indeed, there was a slight decrease of the A-V difference (Fig. 3). Since the blood flow did not change appreciably during this period, this result was consistent with the generally

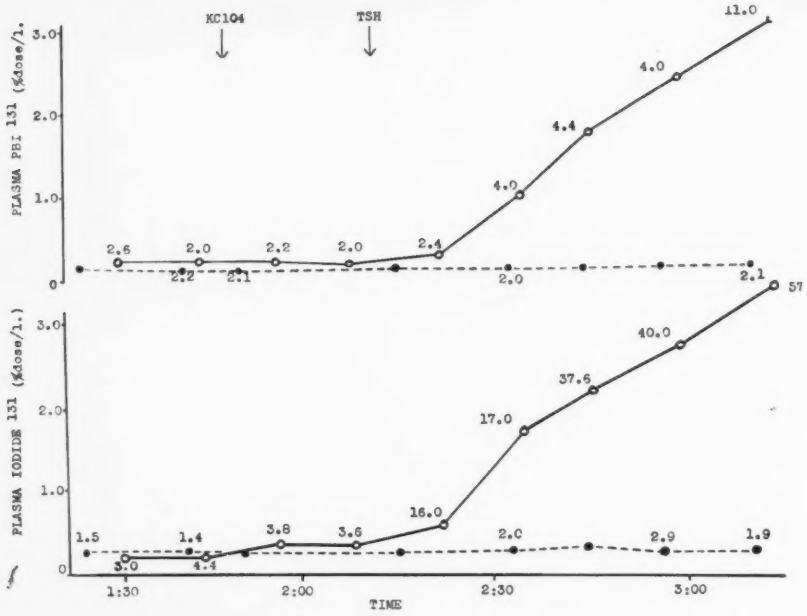

FIG. 2. Effect of thyrotropin in dog pretreated with perchlorate. The tracer dose was given 5 days before the blood samples were obtained, at a time when grandular accumulation was 15% (experiment 8). Injection of potassium perchlorate (100 mg.) led to reversal of the positive arterio-thyroid venous iodide^{131} difference. The injection of TSH (5 U.) was followed by a sustained release from the thyroid of TCA-precipitable and TCA-soluble iodine. Stable iodine concentrations (μg./100 ml.) are indicated by numerals about the corresponding I^{131} points. Increases in the concentration of iodide^{131} and PBI^{131} were apparent in the first thyroid venous blood samples obtained after TSH injection.

accepted view that TSH effects release of thyroidal iodine before changes in the glandular accumulation of iodine are apparent.

Nature of the TCA-soluble I^{131} increment. It is generally believed that in systemic plasma the TCA-soluble iodine is inorganic iodide. Two observations suggested that the TCA-soluble I^{131} in thyroidal venous plasma samples obtained after TSH administration also was largely inorganic iodide.

(1) Anion-exchange resin behavior. The fraction of the total I^{131} which was TCA-precipitable was compared with the value for the proportion of the thyroidal venous plasma I^{131} which was not retained by a column of the anion-exchange resin IR A-400 (acetate form). I^{131} not adsorbed by the resin was determined in several instances by measuring the radioactivity of an aliquot of the effluent from a plasma sample placed on the column and washed through with saline. The agreement between the percentage of TCA-precipitable I^{131} and the percentage of the I^{131} emerging from the

column was good; for example, in a thyroid venous plasma sample obtained late in the TSH period of experiment 7, 13.5% of the I^{131} was TCA-precipitable and 14.9% was not retained by the column.

(2) Behavior of TCA-soluble I^{131} in acid-iodide-iodate solution. The supernatant solution from the TCA precipitation of 2 thyroid venous plasma samples (the last 2 venous samples in experiment 8) was extracted with ether to remove TCA, the aqueous phase was then acidified with HCl, and potassium iodide and iodate were added. After a single extraction of this solution with an equal volume of chloroform, 94% of the I^{131} was found in the chloroform phase. This suggested that the I^{131} had been almost completely oxidized to iodine and hence that the TCA-soluble I^{131} was largely inorganic iodide.

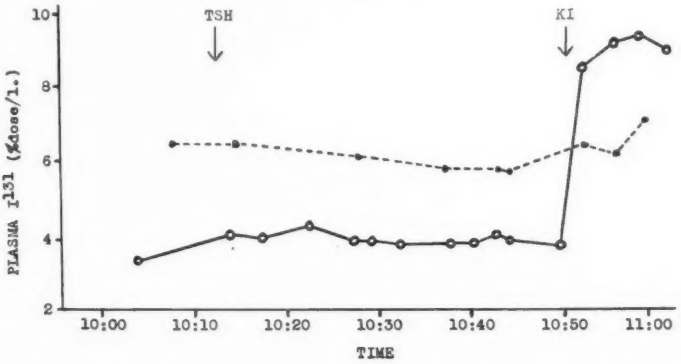

FIG. 3. Effect of thyrotropin on plasma concentration of arterial and thyroid venous iodide^{131}. The experimental animal received the tracer dose of I^{131} 2 hours before the blood samples were obtained. Ordinates represent total plasma I^{131} concentration; TCA-precipitable I^{131} accounted for only a small fraction of the total I^{131} in both arterial and venous samples. The administration of TSH produced no change in thyroidal venous I^{131} concentration; since arterial I^{131} declined slightly during the experimental period, a slight decrease in the A-V difference for plasma iodide occurred. Injection of potassium iodide (84 mg.) effected appreciable release or "wash-out" of I^{131} into the thyroid vein.

Stable iodine. Chemical determination of plasma iodine (total iodine and PBI, "inorganic" iodide by difference) in many of the samples of arterial and of thyroid venous blood was performed (Table 3). In general, as shown in Figures 1 and 2, as well as in Table 3, the values for stable iodide and PBI tended to show changes in the same direction as the I^{131} values. Thus, in experiment 1 the concentration of stable inorganic iodide in thyroid venous plasma increased 8-fold 29 minutes after injection of TSH and the venous PBI increased approximately 3-fold while the corresponding arterial values changed little during the experiment. Surprisingly large increases in the concentration of stable iodide in the thyroid venous plasma were sometimes observed after TSH administration, as in experiment 8,

TABLE 3. PLASMA STABLE IODINE CONCENTRATION AND SPECIFIC ACTIVITY*

Group I—Exp.		Plasma iodine concentration (μg./100 ml.)†					
		Inorganic iodide			PBI		
		Control	TSH	Perchlorate	Control	TSH	Perchlorate
1	A	0.7 (73)	1.0 (50)	1.2 (47)	1.5 (49)	1.0 (67)	1.0 (61)
	V	1.0	1.2	8.0	2.3	3.6	7.6
	(V-A)	0.3	0.2 (27)	6.8 (51)	0.8 (148)	2.6 (79)	6.6 (70)
4	A	—	2.0 (15)	2.5 (14)	—	0.5 (54)	0.5 (48)
	V	—	7.6	7.2	—	5.4	6.0
	(V-A)		5.6 (21)	4.7 (48)		4.9 (67)	5.5 (48)
Group II—Exp.		Control	Perchlorate	TSH	Control	Perchlorate	TSH
5	A	5.0 (7.2)	6.2 (8.4)	5.8 (8.3)	2.0 (4)	1.4 (5)	1.6 (5)
	V	11.4	13.4	—	8.6	7.8	—
	(V-A)	6.4	7.2 (1.0)		6.6 (2.6)	6.4 (1.4)	
7	A	—	5.5 (25)	6.0 (25)	—	1.3 (4.6)	1.3 (4.6)
	V	—	6.5	25.6	—	5.8	17.2
	(V-A)		1.0 (32)	19.6 (8.5)		4.5 (3.1)	15.9 (8.4)
8	A	1.4 (17)	1.5 (18)	2.4 (11)	2.2 (7.7)	2.1 (6.7)	2.1 (7.2)
	V	3.7	3.7	57	2.3	2.1	11.0
	(V-A)	2.3	2.2 (2.7)	54.6 (5.9)	0.1 (40)	0	8.9 (34)

* The values of specific activity of arterial iodide and PBI and of the iodide and PBI released by the gland (the V-A difference) are in parentheses, and are expressed as % dose I^{131} per mg. iodine.
† Plasma iodine concentrations are those observed at the end of the corresponding experimental periods.

in which the veno-arterial (V-A) concentration difference for iodide at the end of the TSH period was 54.6 ug./100 ml., greatly exceeding the corresponding V-A value for PBI^{127} (Table 3). An unexpected finding was that the concentration of stable iodide in thyroid venous plasma during control periods before perchlorate or TSH administration might be greater than the arterial value (Experiments, 1, 5, 8). Since the corresponding I^{131} values tended to show either a positive or no arterio-venous difference, this suggested the possibility of thyroid release of iodide of specific activity lower than that of the arterial iodide. Calculation from the stable and radioactive iodine data of the specific activities of the veno-arterial differences of PBI and inorganic I did not lead to consistent results but certain observations seemed of interest (Table 3). Thus, the specific activity of the iodide released from the gland (V-A, iodide) after administration of TSH and perchlorate tended to resemble the specific activity of the PBI increment (V-A, PBI), both in value and in direction of change from the arterial iodide and PBI specific activity (Experiments 1, 4, 7); the notable exception was experiment 8, where the specific activity of the venous iodide increment after TSH injection was only one-sixth that of the PBI increment, the disparity being perhaps entirely accounted for by the unusually high specific activity of the PBI, since a rather small secretion of PBI^{127} occurred after TSH as compared with PBI^{131}. Another interesting feature is that in one experiment (no. 4) the iodide released from the gland after TSH was of higher specific activity than the arterial iodide.

Many biologic factors could affect the specific activity of the thyroidal

secretory products, such as the iodine content of the gland, the previous diet, the degree of glandular activity, the plasma iodide concentration and possibly variations in the proportion of thyroxine and triiodothyronine secreted, both amino acids having been detected in the thyroid venous plasma of several animal species (9); in addition, the analytic error in estimating the inorganic stable iodide (and hence specific activity) from a small difference between total iodine and PBI may also contribute to the difficulty in interpreting the specific activity data.

Blood flow. The values for blood flow recorded in Tables 1 and 2 represent the average value in each experiment of the thyroid venous blood collected per minute; the range was between 0.22 and 0.73 ml./min. Although this undoubtedly did not represent the entire venous outflow from the hemithyroid it did provide some indication, in successive samples, of the constancy of flow. Variations in flow were encountered but they were small compared to the effects induced by TSH upon the concentration of iodide^{131} and PBI^{131}. When a decrease in thyroid venous blood flow occurred during the experiment, the veno-arterial concentration differences for both PBI^{131} and iodide^{131} tended to increase, a finding supporting Sodeberg's observation that the rate of hormonal secretion is independent of blood flow (4). No definite early effect of TSH on thyroid blood flow was noted. Assuming that the blood flows recorded in Tables 1 and 2 were complete flows, that the hematocrit was 50% and that the average veno-arterial iodide^{131} concentration difference throughout the TSH-perchlorate period was one-half the value at the end of the period, one may calculate that in experiment 1, 0.038% of the dose was secreted per hour as iodide^{131} during the TSH period, and 0.051% as PBI^{131}; this corresponds to 0.25% and 0.33% of the hemi-glandular I^{131} secreted as iodide^{131} and PBI^{131} per hour, differences which would be very difficult to detect by external counting techniques.

DISCUSSION

The results suggest that the administration of TSH led to a prompt release from the thyroid not only of hormonal iodine but also of inorganic iodide. The magnitude and the time-course of the two were comparable, and changes in PBI and inorganic iodide concentrations were apparent within 30 minutes. The iodide released from the gland in response to TSH is probably iodine which has been previously organically bound.

It is unlikely that iodide newly entering the thyroid from the circulation could contribute greatly to the glandular secretion of iodide induced by thyrotropin. In studies of A-V iodide differences of the unblocked, unstimulated gland the response to the administration of perchlorate indicated that relatively little iodide newly entered from the blood was present within the gland (5). When glandular iodine was minimally labelled, the administration of TSH had little early effect on the net thyroidal accumu-

lation of plasma iodide, as judged by the absence of appreciable change in the arterio-thyroid venous iodide^{131} difference during a 40-minute period after TSH injection, while a prompt release of labelled iodide occurred in similar experiments in which the iodine in the gland had been labelled.

Although administered TSH did not lead to a prompt increase in glandular accumulation of plasma iodide, it is conceivable that the observed effect on glandular release of iodide might result from a temporary impairment of organic binding of iodide by TSH. If this were so, and organic binding were the rate-determining step in hormone synthesis, the injection of perchlorate might then lead to glandular expulsion of an appreciable quantity of newly-entered iodide, as has been seen in experimental situations where organic binding has been impaired by anti-thyroid drugs, such as propylthiouracil (10). However, the hypothesis that TSH might exert a temporary inhibitory effect on organic binding seems unlikely for several reasons. (a) The release of labelled iodide from the gland consequent to TSH injection was detectable even in the absence of perchlorate. (b) When net glandular accumulation of iodide^{131} from the plasma was prevented by pretreating the dogs with perchlorate (group II experiments) the intraglandular content of newly-entered iodide must have been small; yet such pretreatment did not prevent release of iodide when TSH was given. (c) In at least one experiment (#4), the iodide liberated from the gland after TSH injection was of higher specific activity than the arterial iodide, so that even if the latter had contributed to the iodide released there must have been a contribution from another intraglandular source as well.

These considerations thus suggest that the iodide which left the gland when TSH was given originated within the gland. The pattern of discharge of labelled iodide after TSH administration was one of progressive increase in the venous iodide concentration rather than of abrupt liberation of iodide such as might be expected if diffusion of a quantity of glandular iodine into the venous circulation were suddenly permitted to occur. The release pattern observed thus did not resemble the exponential discharge of labelled iodine that has been encountered both by external counting techniques in man (11) and by thyroid venous measurements in the dog (12) when organic binding was inhibited before perchlorate or thiocyanate was given. It seems likely from the character of the iodide release curve that TSH led to the production of an increased quantity of inorganic iodide within the gland from an organic iodine precursor, rather than that TSH had the effect of facilitating diffusion from an intrathyroidal compartment of preformed iodide. There is a suggestion from the present studies that the iodide-trapping mechanism of the gland may lead to some retention of the iodide formed within the gland since larger increments of thyroidal venous iodide were found when perchlorate was given after TSH had been injected than with TSH alone.

A number of published observations support, or are at least consistent

with, the view that thyrotropin may lead to an enhanced release of iodide from the thyroid gland. Stanbury and associates studied endemic, iodine-deficient goiter, a condition in which it is probable that the thyroid is greatly stimulated by endogenous TSH; when organic binding of iodine was blocked by administering antithyroid drug to goitrous subjects whose glands had previously been labelled with I^{131}, there was an increase in the urinary excretion of iodine which was larger than could be explained on the basis of a block to the re-utilization of iodide formed from peripheral degradation of hormone. They suggested that the intraglandular deiodination of organic iodine compounds might be the source of the extra urinary iodine (13). Becker and co-workers (14) observed that the urinary excretion of labelled iodide rose appreciably within 24 hours of the administration of TSH to euthyroid and hyperthyroid subjects who had received a tracer dose of I^{131} and who were then given thiocyanate to prevent further accumulation of iodide. They observed that the increased urinary iodine^{131} excretion could not be explained solely on the basis of degradation of labelled circulating hormone and suggested that the excess urinary iodine might reflect a TSH-induced increase in the serum concentration of an iodine-containing compound which was rapidly cleared by the kidney. The enhancing effect of TSH on urinary I^{131} excretion in persons in whom the thyroid gland has been previously isotopically labelled has been confirmed in this laboratory in several euthyroid and one hyperthyroid subject; additional observations have shown that the enhanced urinary excretion can be demonstrated within 6 hours of the intramuscular administration of TSH (15). Recently, Deiss and associates have studied the effects of TSH on the rate of disappearance of plasma iodide^{131} from the systemic circulation and have obtained results consistent with the view that an early effect of TSH is stimulation of the discharge of iodide from the gland (16). Halmi (17) noted that the thyroid/serum iodide^{131} concentration ratio decreased within a few hours after TSH administration in the hypophysectomized rat, and suggested as a possible explanation the release of iodide from deiodination of iodotyrosines released during TSH-induced hormonal secretion. Adams and Purves (18) have stated that the increase in plasma I^{131} concentration observed three hours after TSH administration to guinea pigs whose glandular iodine has been labelled is due not only to an increase in PBI^{131} but also in TCA-soluble I^{131}.

The possible existence of a small "second" pool of iodide within the thyroid gland has been suggested by Hickey and Brownell (19) as an explanation for unexpectedly high urinary I^{131} excretion values observed during the first and second weeks after I^{131} administration in man. Slingerland and his associates (20) have also indicated that significant quantities of iodide may be secreted from the thyroid gland. Nadler and Leblond (21) have claimed that some iodide leaves the thyroid gland in the rat, and that 50% of the iodide so released may be derived from deiodination

reactions in the gland. Studies in this laboratory of the iodide^{131} concentration in arterial and thyroid venous plasma of the dog have also indicated the presence within the gland of iodide which had not newly entered from the circulation; this iodide, presumably derived from an intraglandular organic precursor, was released from the gland when the mechanism for maintaining a thyroid-plasma iodide concentration gradient was rendered inoperative by administering perchlorate (5).

The precursor or source of the iodide which appears within the thyroid gland and is discharged into thyroidal venous blood in response to the administration of TSH is not known. It seems reasonable that the iodide may be derived from deiodination of thyroidal organic iodine compounds, since the occurrence of deiodinating enzymes within the gland is well known (22), and particularly since thyroidal dehalogenase activity is said to be enhanced by TSH administration (23). This view is compatible with the observations that in the gland whose iodine stores were unlabelled, no evidence of TSH-induced release of labelled iodide was apparent, while such release was observed when organic binding of labelled iodine had been appreciable, and also with the observations that the specific activity of the iodide and PBI increments were often comparable. Whether the enhanced hormonal secretion and release of iodide are independent effects of TSH which occur *pari passu* or whether one or the other is the primary effect of TSH and the other a consequence is not clear. It is also not known whether the iodide which appears within the gland and is released under the influence of TSH and perchlorate is freely miscible with iodide entering the gland from the plasma or whether it enters a separate iodide pool.

The physiological implications of the release of iodide from the thyroid as a response to TSH remain to be explored, as well as the duration of the effect and its relationship to the well-known later effect of TSH on enhancing thyroidal accumulation of iodine. The demonstration that iodide release can occur may affect the validity of certain assumptions that are sometimes made—for example, that thyroidal release of I^{131} may be equated with hormonal secretion, and that the specific activity of intraglandular iodide and of plasma iodide are equal. The results strongly support the views of Roche and Michel (22) that intraglandular deiodinase mechanisms may be physiologically important.

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ADRENAL CORTICAL AND MEDULLARY RESPONSES TO TRAUMA IN DOGS WITH ISOLATED PITUITARIES[1]

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ABSTRACT

The adrenal venous output of 17-hydroxycorticosteroids and catechol amines was measured in the basal state and following burn trauma in a series of dogs with their brains removed. The brain above the inferior colliculus was removed, leaving the pituitary in situ. For as long as five days after brain removal, such animals revealed high resting levels of corticoids and two-thirds of the animals responded to burn trauma with increased outputs of corticosteroids. Low resting levels of catechol amine output were observed in the dogs with isolated pituitaries, with normal increased response following burns.

WE ARE presently passing through what might be called the era of "centers" in the field of neuroendocrinology. A large and convincing literature confirms the original observations of Hume (1) and Harris (2) with regard to the importance of the hypothalamus in pituitary-adrenal function. In similar fashion, the pioneer studies of Cannon (3) and Hess (4) have been extended by recent workers, and emphasize the relationship of various CNS areas to adrenal medullary secretion. Recent observations by several different groups of investigators (5, 6, 7), strongly suggest the participation of the cerebral cortex and limbic system in both excitatory and inhibitory aspects of adrenal cortical function.

Evidence has not previously been available with which to evaluate the true role of the hypothesized "centers" in these various activities. It is conceivable that destruction of specific controlling areas such as the hypothalamus results in absent target organ responses to trauma, not because of removal of the single stimulatory pathway, but rather because other stimulatory areas continue to be inhibited by higher centers. We found in previous preliminary experiments (8) that removal of the brain down to the hypothalamus did not abolish the normal 17-hydroxycorticosteroid output increase from the adrenal cortex in response to operative trauma. In the first preliminary experiments blood levels of corticosteroids fell after removal of the hypothalamus. A few additional preliminary experiments (9), however, revealed persistence of elevated corticosteroid secretion for as long as six hours following the removal of large portions of the hypothalamus in some animals. Histological studies were not reported in

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these preparations, and the present studies were carried out to determine the effect of complete "isolation" of the pituitary from all nerve impulses. Portions of this work have been presented in abstract form (10).

It was felt desirable to determine both adrenal cortical and medullary function in dogs with isolated pituitaries. According to current concepts, such a preparation would not be expected to demonstrate pituitary ACTH release in response to trauma. In addition, there is no experimental background on which to base a prediction as to the ACTH-secreting behavior of such an animal in the basal or "resting" state. There is also little evidence on which to predict the adequacy of adrenal medullary response in such animals, both with respect to the "resting" state and activation following trauma. The purpose of the present study was to provide the data whereby these questions could be answered.

METHODS

Healthy adult mongrel dogs ranging in weight from 12 to 20 kg. were used for these studies. Sterile technique was carried out in all surgical operations, and intravenous sodium pentobarbital, 30 mg./kg., was the anesthetic agent. Adrenal venous cannulations were performed according to the technique of Hume and Nelson (11). It is possible with this preparation to obtain intermittent samples of adrenal venous blood in both the acute and chronic state, permitting sample collections in the untraumatized, unaesthetized dog for as long as several weeks following surgery. 17-hydroxycorticosteroid content of timed samples of adrenal venous blood were determined by the method of Nelson and Samuels (12). Assay of cavernous sinus blood ACTH content (13) was performed according to the method of Nelson and Hume (14). Epinephrine and norepinephrine were determined by the Aranow modification (15) of the method of Weil-Malherbe and Bone (16), and the values are added together in each sample making one figure for total catechol amines (E-NE). Blood pressures were taken by means of an indwelling plastic cannula in the femoral artery connected to a mercury manometer. Rectal temperatures were measured with a recording thermometer inserted at least three inches into the rectum.

Brain removal was performed using a standard neurosurgical technique. A craniotomy was first carried out using a one inch trephine, followed by removal of most of the skull over the cerebral cortex with a double-action rongeur. Control of venous bleeding from the skull was easily achieved with bone wax, and care was taken not to injure the longitudinal sinus. After attention to hemostasis, the dura was opened parallel to the longitudinal sinus and approximately 1 mm. from it. Small bridging veins from the cortex into the sinus were cauterized. Early in the development of techniques for brain removal, the procedure was performed slowly, with great attention to hemostasis after removal of each layer. Twenty-four hour survival percentage was low with this method (20% to 50%), and gradually a method was worked out in which complete removal of the brain down to the pituitary, with transection of the brain stem at the level of the inferior colliculus, could be performed within one to two minutes after incision of the dura. This is accomplished by using a large suction tubing to remove brain down to the desired level on both sides. The middle cerebral arteries persist as thick vascular strands following this maneuver, and silver clips are placed at their origins. It is then possible to perform, easily and in a relatively bloodless field, a more thorough cleaning of the diaphragma sella with a fine suction tip. In addition, in several experiments the basilar artery was transected by cutting between two silver clips placed on it.

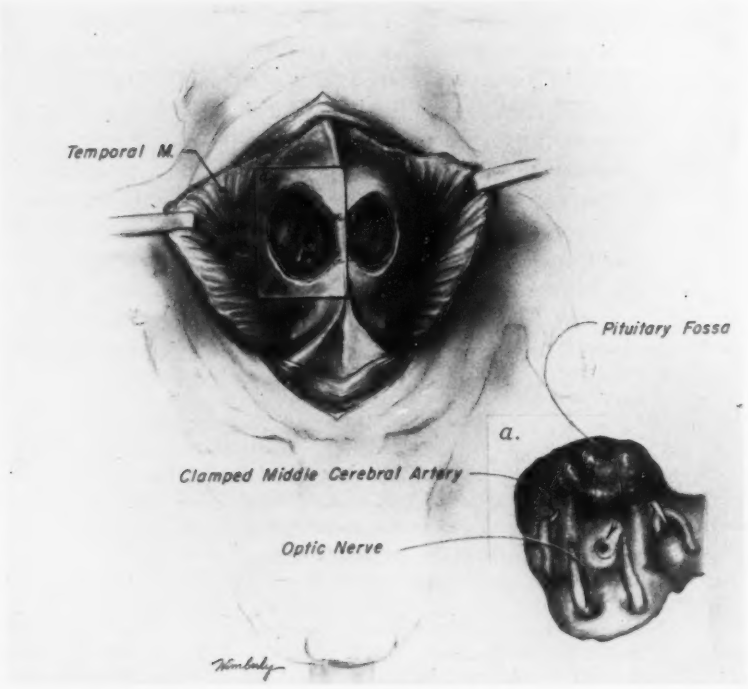

FIG. 1. Artist's sketch of completed brain removal procedure. A bilateral craniotomy has been performed, with suction removal of brain tissue down to diaphragma sella and inferior colliculus. The fibrous diaphragma sella, attached firmly to the anterior and posterior clinoids, protects the pituitary from suction removal. Suction tip must be inserted through orifice in diaphragm to insure complete removal of median eminence.

Figure 1 illustrates the appearance of the skull following brain removal, and it can be seen from the insert that considerable attention is given to completely cleaning the relatively tough diaphragma sella of all residual tissue. At this juncture it is decided whether to make a preparation in which the small remnant of median eminence which remains below the diaphragma sella is to be removed. In order to do this, it is necessary to insert a small curved suction tip through the stalk orifice in the diaphragma. The principal danger in such a maneuver is the possibility of devascularizing the pituitary. The anterior pituitary of the dog receives its blood supply from two sources, one passing from the posterior pituitary and the other a small branch on each side from the internal carotid. Occasionally these arteries pass up almost to the inferior surface of the diaphragm before entering the pituitary, and in those instances, suction removal of the median eminence will result in the destruction of the pituitary blood supply, in addition to the hematoma which often forms. Therefore, in many experiments, no attempt was made to remove the sub-diaphragmatic fragment of the median eminence completely. After the preparation was completed, small pledgets of oxidized cellulose were placed over the diaphragma sella, and on the transected brain stem itself. The temporal muscles were reapproximated with interrupted chromic catgut, and the galea aponeurotica was closed with a running 3-0 chromic catgut suture. A subcuticular suture of 4-0 chromic catgut

completed the closure. At necropsy the whole heads were placed in 10% formalin. Seven to ten days later the pituitary remnant and hind brain segment were removed. Because of the absence of higher structures, it is easy to remove the diaphragma sella and sella turcica contents intact. Serial sagittal sections 5 to 6 μ thick were made of the pituitaries. Sections were alternatively stained with the Gomori and haematoxylin and eosin technique. Serial sagittal sections were made of the hind brain and were stained with haematoxylin and eosin.

Blood loss was carefully measured and quantitatively replaced with freshly drawn blood. Urine outputs were measured in most animals and losses were replaced with equal parts of 5% glucose and 5% glucose in saline. It is extremely important to give intensive nursing care to these animals because they are very prone to develop pneumonia. Endotracheal suction was performed at frequent intervals, and in an occasional animal with slow and relatively shallow respiration, the endotracheal tube was connected to a respirator and a few deep breaths every hour were given to the dog. The animals were turned every three hours from one side to the other, and in a few experiments norepinephrine was given to maintain a falling systolic blood pressure.

In one experiment, all structures of the hind leg were transected except for an artery, vein and nerve as has previously been described (17). In another animal, a bilateral cervical sympathectomy was performed. The traumatic stimulus used in these studies was a five minute burn of the paw and lower foot. It has previously been determined in our laboratories that such a burn, resulting in tissue necrosis and nerve stimulation, leads to a marked adrenal cortical response in 100% of the animals so burned. After such burns the animals do not manifest a further increase in adrenal cortical output when given exogenous corticotrophin (ACTH). The burn was applied with a large infrared lamp which was held approximately 1 cm. from the area to be burned. At the end of each experimental period 25 units of ACTH were given intravenously in order to produce adrenal cortical stimulation to compare with the experimental findings.

RESULTS

We have thus far been unable to obtain survival for more than five days in dogs with brains removed to the level of the inferior colliculus. Such animals follow a fairly predictable course: Immediately following removal of the brain there is a blood pressure fall of between 5 and 15 mm. of mercury. The pressure becomes stabilized at this level for some hours and between 12 and 24 hours later begins a gradual drift downward which is delayed temporarily if a vasoconstrictor such as norepinephrine is given. The blood pressure fall occurs despite good nursing care and attention to fluid and blood replacement. Attempts are under way at the present time to increase survival times by staged de-encephalization several days apart. Brainless dogs often raise their heads and howl when the extremity is burned, although no thalamus or cerebral cortex is present. In addition, they make occasional intermittent movements of both fore and hindquarters. The animals are completely poikilothermic and attention must be given to maintenance of the correct body temperature, either by heating blankets or exposure to a cool environment, whichever is appropriate.

The first series of experiments in which adrenal cortical function only was determined in dogs with isolated pituitaries is presented in Table 1. The data from two control dogs, 18 hours after adrenal vein cannulation,

are presented to contrast the normal response with that observed in dogs with isolated pituitaries. The control dogs have very low values for basal corticosteroid output, as do a series of similar control animals with craniotomy and dural incision, but intact brains, on the day preceding sampling. Five to ten minutes after burn trauma, the anesthetized control dogs have marked adrenal cortical responses which are not further increased by exogenous ACTH. In contrast to this, all dogs with isolated pituitaries had high resting levels of adrenal cortical secretion unless pituitary infarction had occurred. In response to burn trauma, most of the animals presented in Table 1 had a significant adrenal corticosteroid response above control levels. Adrenal venous blood flows, ranging from 1.0 to 4.2 cc./minute, usually were not significantly altered following burn trauma.

TABLE 1. RIGHT ADRENAL VENOUS 17-HYDROXYCORTICOSTEROID OUTPUT PER MINUTE (μG.) BEFORE AND AFTER TRAUMA (FIVE MINUTE BURN) AND FOLLOWING I.V. ACTH

Dogs with brain removal were unanesthetized, rectal temperatures varied from 35–39° C, and blood pressures ranged from 70 to 110 mm. All figures represent average of two or more determinations taken within 10 minutes of each other. Control dogs were burned 18 hours after adrenal vein cannulation, and were anesthetized prior to burning.

Experiment number	Normal control #1	Normal control #2	B-36	B-47	B-53	B-58	B-61	B-62	B-70	B-71†	B-100*	B-120	B-123	B-135
Values before trauma	1.2	0.2	6.8	4.8	7.2	11.4	16.1	7.4	6.1	6.4	6.3	8.5	—	4.6
5–10 minutes after trauma	26.8	14.9	16.9	11.4	9.8	13.4	15.9	11.9	10.0	9.4	11.1	—	10.9	10.8
1 hour after trauma	20.2	7.6	15.0	12.2	8.8	—	12.2	10.0	8.0	7.2	—	—	6.2	5.2
5 minutes after 25 units I.V. ACTH	28.2	15.6	—	—	15.4	13.6	12.3	15.6	14.4	11.8	17.0	14.6	—	22.6
Number of hours after brain removal trauma performed	—	—	120	26	4	9	25	6	29	26	27	24	48	27

* Isolated leg preparation.
† Bilateral cervical sympathectomy 2 weeks before brain removal.

Animal B-61 failed to demonstrate an elevation of adrenal corticosteroid output following burn, but appeared to have already reached high levels before burning. In several instances the levels fell markedly one hour after trauma. In the majority of cases, administration of exogenous ACTH resulted in a further increase of adrenal cortical secretion as compared to that observed following burn trauma.

Figure 2 illustrates in more detail the pattern of adrenal cortical function in Dog B-36 following brain removal. Five days after the brain had been removed, the dog still demonstrated a high resting secretion, ranging from 7 to 9 μg. per minute. Following the burn, output rose to 16.8 μg. per minute and remained in the high range for several hours. In Figure 3 may be seen the results of an experiment in which the animal was burned 26 hours following brain removal. Corticosteroid outputs immediately rose following the burn from between 4 and 5 μg. per minute to over 14 μg. per minute. Response to ACTH on the day of adrenal vein cannulation

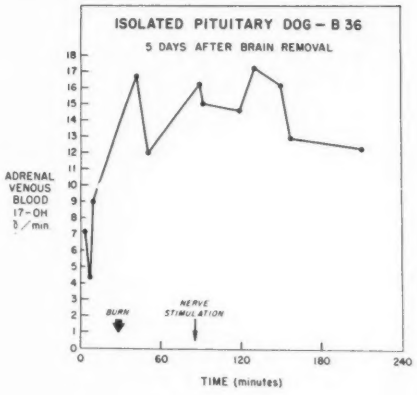

FIG. 2. Dog (B-36). Five days after brain removal adrenal venous corticosteroid outputs ranged from seven to nine micrograms per minute, with further increase to 16.8 micrograms following burn.

was 25.8 μg. per minute, a considerably greater value than that observed following burning after pituitary isolation.

Figure 4 illustrates an experiment in which the animal was burned several times, with a significant response following each burn. The brain had been removed six hours prior to the burn in this experiment and adrenal corticosteroid outputs had fallen to levels ranging between two and five μg. per minute. The levels immediately rose following burning, to between 8 and 10 μg. per minute. Another burn resulted in a maximal output of almost 18 μg. per minute, and three succeeding burns each resulted in an increase in the adrenal corticosteroid output to similar levels. Exogenous ACTH did not result in a greater corticosteroid output than that observed following burns.

Figure 5 illustrates the effect of a burn in an animal with combined isolated pituitary and isolated leg. When the isolated nerve was intact and the leg was burned, resting levels were so high that no response was observed. The nerve was then cut and the levels fell to a value of 7 μg. per minute. At this time, the isolated leg was again burned and no response was observed. The normal leg was then burned and an immediate response

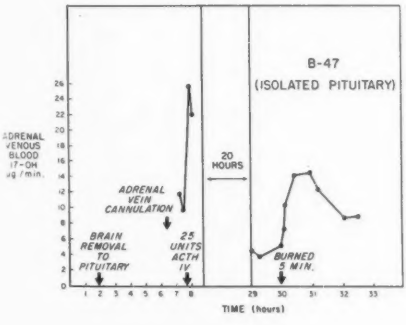

FIG. 3. Dog (B-47). 26 hours after brain removal animal responds to burn with elevated corticosteroid output. Note high resting levels of corticosteroid output and failure of burn to achieve an adrenal response comparable to that observed following exogenous ACTH.

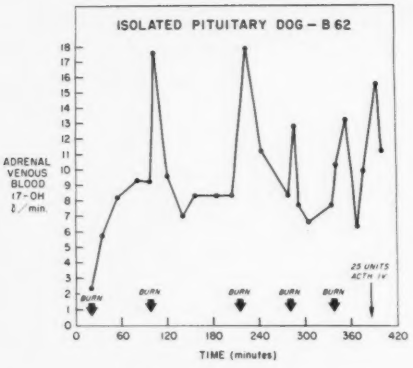

FIG. 4. Dog (B-62). Resting level of corticosteroid output in this animal ranged from 2.2 to 5 gm. per minute. Burns on five different occasions resulted in sharp increases in corticosteroid output. The increase in output following burning was comparable to that obtained following exogenous ACTH.

to between 13 and 14 μg. was observed. Exogenous ACTH resulted in a further small increment of increase in corticosteroid output.

Table 2 gives results in which epinephrine and norepinephrine outputs were determined in addition to corticosteroids. It may be seen that dog B-64 had neither high resting levels of adrenal corticosteroid output nor response to trauma, and represents the only animal without high resting levels observed in this series. At necropsy the pituitary was found to be completely infarcted. All other animals had high resting corticosteroid outputs, with approximately half showing significant adrenal responses following burning. One experiment in this series is illustrated in Figure 6 (B-59). After cannulation control levels ranged from 2.5 to 5.5 μg. per minute. After burning, the secretion rose to 10.8 μg. per minute. Hypophysectomy was then performed and the output fell to near zero and remained there until exogenous ACTH was given, resulting in a response similar to that seen following burning of the leg. Also in this experiment, cavernous sinus ACTH content was assayed after burning with the pituitary intact, and following hypophysectomy. It may be seen that there was an amount of ACTH in the cavernous sinus blood equivalent to that ob-

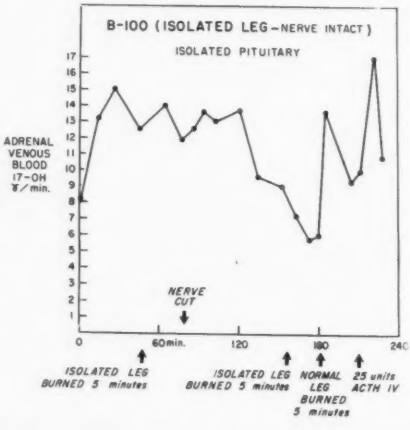

FIG. 5. Dog (B-100). Burning of the isolated innervated leg resulted in no further increase from very high resting corticosteroid levels. After nerve transection, levels declined and reburning of the isolated leg resulted in no increase in corticosteroid output. There was an immediate increase in corticosteroid output following burning of the other normally innervated leg, with further increase following exogenous ACTH.

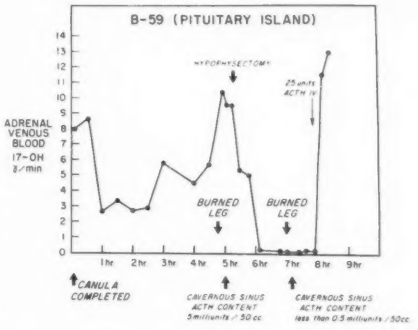
B-59 (PITUITARY ISLAND)
HYPOPHYSECTOMY
25 units ACTH IV
ADRENAL VENOUS BLOOD 17-OH γ/min
BURNED LEG
BURNED LEG
1hr. 2hr. 3hr. 4hr. 5hr. 6hr. 7hr. 8hr. 9hr.
CANULA COMPLETED
CAVERNOUS SINUS ACTH CONTENT 5 milliunits / 50 cc.
CAVERNOUS SINUS ACTH CONTENT less than 0.5 milliunits / 50cc.

FIG. 6. Dog (B-59). Burn resulted in an immediate and marked increase in corticosteroid output accompanied by a significant cavernous sinus ACTH content. Following hypophysectomy there was no adrenal venous corticosteroid response nor ACTH present in cavernous sinus blood.

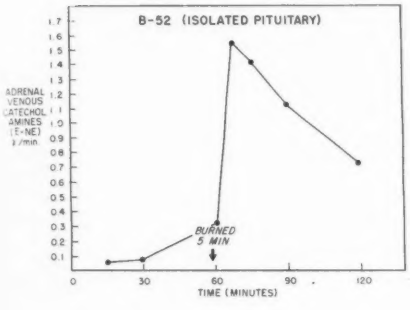

FIG. 7. Dog (B-52). Burn in this animal resulted in immediate elevation of adrenal venous catechol amine output to very high levels.

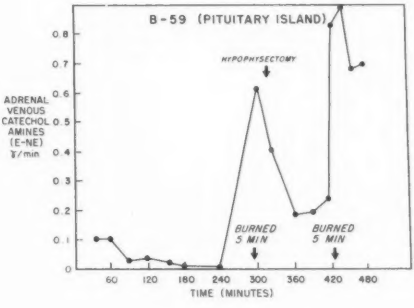

FIG. 8. Dog (B-69). Burning resulted in a rapid elevation of adrenal venous catechol amine output on two separate occasions, the second following hypophysectomy.

served in normal animals following burn trauma, but that following hypophysectomy, no ACTH was measurable.

All animals of this series had a marked adrenal medullary response following burning. The output in most instances tended to return toward normal one hour following the burn. Two of these experiments are illustrated in Figures 7 and 8. Figure 7 (B-52), portrays an animal which was burned with a control level of 0.08 μg. E-NE per minute, with a return toward basal levels in the succeeding hour. In Figure 8 may be seen a similar experiment in which a burn resulted in a marked response in the dog with isolated pituitary, with a similar response after hypophysectomy.

Eight animals of the total series of dogs reported in Tables 1 and 2 had no remaining median eminence fragment. These were dogs B-52, B-62,

TABLE 2. RIGHT ADRENAL VENOUS 17-HYDROXYCORTICOSTEROID AND TOTAL CATECHOL AMINES E-NE OUPUT PER MINUTE (μG.) BEFORE AND AFTER TRAUMA (FIVE MINUTE BURN) AND FOLLOWING I.V. ACTH

Animals with brain removal were unanesthetized, rectal temperatures varied from 35–39° C, and blood pressures ranged from 70 to 110 mm. All figures represent average of two or more determinations taken within 10 minutes of each other.

Experiment number	B-43		B-46		B-52		B-59†		B-64*		B-75		B-86		B-114	
	17-OH	E-NE	17-OH	E-NE	17-OH	E-NE	17-OH	E-NE	17-OH	E-NE	17-OH	E-NE	17-OH	E-NE	17-OH	E-NE
Values before trauma	4.0	0.10	—	0.14	08.4	0.06	5.3	0.01	3.2	0.08	7.2	0.21	7.7	0.56	10.3	0.37
5–10 minutes after trauma	8.7	0.60	13.3	2.66	7.1	1.5	9.8	0.60	2.4	1.10	7.7	0.61	9.1	2.67	10.4	1.70
1 hour after trauma	—	—	9.8	1.40	6.8	0.7	—	0.40	3.2	0.14	7.1	—	7.4	0.74	4.0	1.40
5 minutes after 25 units ACTH I.V.	15.1	—	—	—	9.2	—	12.0	—	24.8	—	14.1	—	—	—	—	—
Number of hours after brain removal trauma performed	48		25		24		24		27		22		24		24	

* Pituitary Infarct.
† Dog responded a second time with increased E-NE output.

FIG. 9. Pituitary, Dog B-59 (13.5×). This section shows optic nerve, anterior and posterior pituitary and diaphragma sella. It is clear that a sub-sellar remnant of the median eminence of the hypothalamus persists in this section.

B-70, B-71, B-86, B-100, B-120 and B-135. All other dogs reported in this series revealed fragments of median eminence in one or more of the slides. All such tissue was, of course, located below the orifice in the diaphragma sella. Figures 9 and 10 show the persisting anatomical structures in the sella turcica and hind brain area in dog B-59. It can be seen that a small remnant of median eminence is present, although this fragment is clearly

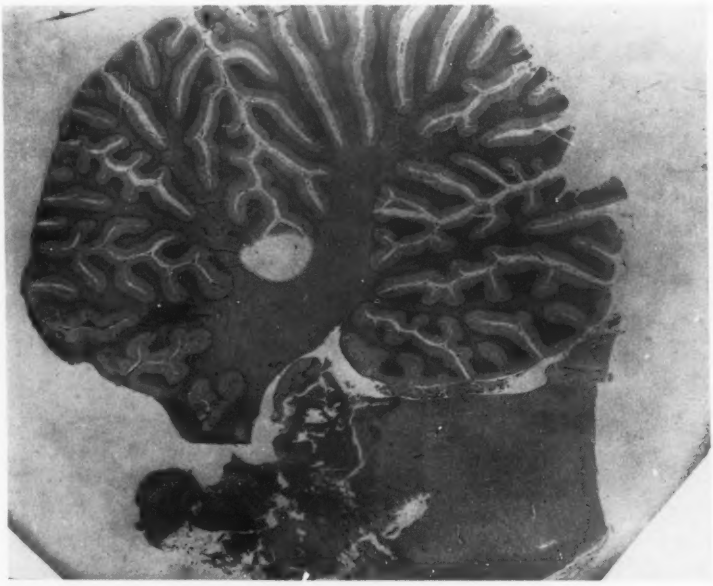

FIG. 10. Hind brain, Dog B-59 (13.5×). Removal of the brain has been carried out down to the mid-pons.

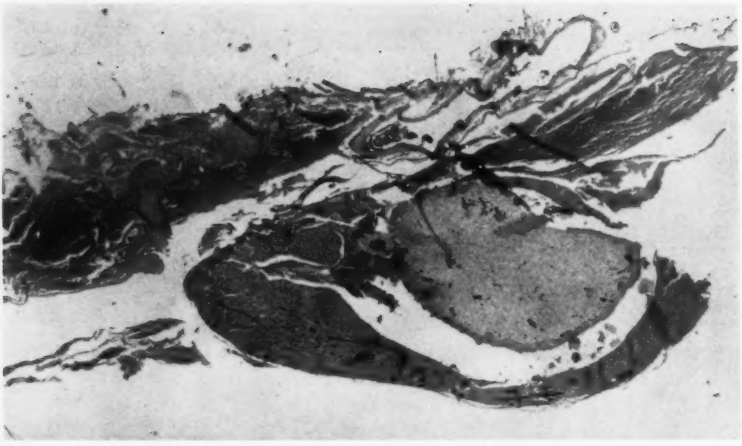

FIG. 11. Pituitary, Dog B-62, (13.5×). No median eminence fragment is present. Special attention was given to suction removal of this area through diaphragmatic orifice.

below the diaphragm. Sections of the remaining hind brain reveal absence of all brain tissue cranial to the mid portion of the pons. Figures 11 and 12 illustrate the pituitary and hind brain areas of dog B-62. No median eminence is present in the pituitary sections, and hind brain sections

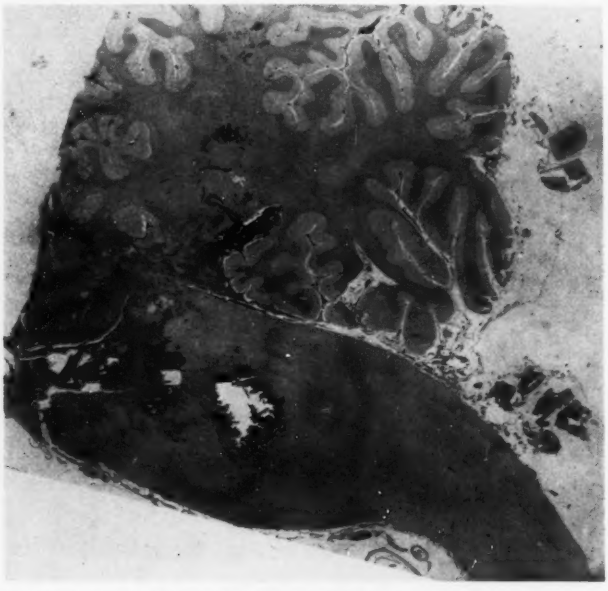

FIG. 12. Hind brain, Dog B-62 (13.5×). The section shows removal of the brain up to and including the rostral pons.

TABLE 3. COMPARISON OF ADRENAL VENOUS AND PERIPHERAL BLOOD 17-HYDROXYCORTICOSTEROID VALUES BEFORE AND 24 HOURS AFTER BRAIN REMOVAL

Experiment Number		Peripheral μg. % Plasma	Adrenal μg./minute
B 121	Before	6.1	5.8
	After	14.6	15.2
B 125	Before	5.3	4.8
	After	14.4	14.2

showed brain removal to extend down to and include the cranial portion of the pons.

Table 3 shows a comparison of adrenal venous and peripheral blood 17-hydroxycorticosteroid values before and after brain removal. In both animals, control samples were obtained two hours after adrenal vein cannulation. Twenty-four hours after brain removal high resting levels were observed in both peripheral and adrenal venous blood. The reason these experiments are not reported in Tables 1 or 2 is that few blood samples were obtained, and burns and ACTH administration were not carried out.

DISCUSSION

High resting corticosteroid levels were observed in all animals with brains removed down to the pituitary, providing the pituitary was not devascularized by the surgical procedure. This is in marked contrast to the consistently low basal values for corticosteroid output seen in the normal unanesthetized animal. The elevation of basal secretion is not of comparable magnitude to that observed following exogenous ACTH. A similar finding has been observed in decorticated, unanesthetized dogs (18) and will be presented in detail elsewhere.

There are at least two explanations which may be presented to explain the elevated basal output in dogs with isolated pituitaries. A humoral inhibitory factor might be produced by higher central nervous system areas such as the cerebral cortex which acts directly on the pituitary. Removal of these higher centers leaves the uninhibited pituitary with an increased rate of ACTH release. The implication of this hypothesis is that the pituitary is capable of spontaneous ACTH release after removal of humoral inhibition. An alternative hypothesis is that higher central nervous system areas inhibit an ACTH-releasing center located elsewhere. Removal of this inhibition results in the accelerated release of a neurohumor which passes to the pituitary resulting in ACTH secretion. Inasmuch as high basal levels are observed in the unstimulated dogs with isolated pituitaries, this would imply a continuing series of stimulatory impulses to the area producing the ACTH-releasing substance. High central nervous system inhibition could be either nervous or humoral if it acted on a hypothalamic or other ACTH releasing area, but would necessarily be humoral if the pituitary was the site of action.

In addition to high resting adrenal cortical secretion, following burning dogs with isolated pituitaries demonstrate ACTH concentrations in cavernous sinus blood comparable to normal normals, and have significant 17-hydroxycorticosteroid secretion in adrenal venous blood. The post-stimulatory changes in adrenal venous output per minute of corticosteroids occur in the absence of significant changes in adrenal blood flow. One of the several possible explanations for pituitary ACTH release following burns in dogs with isolated pituitaries is the persistence of a direct innervation of the anterior pituitary (19, 20). The consensus of workers in the field appears to be that there is no innervation of the anterior pituitary, either by the sympathetic or parasympathetic nervous system (21, 22). Recent electron microscopy studies (23) of the anterior pituitary reveal an absence of nerve fibers. In one animal of this study, B-71, bilateral cervical sympathectomy did not prevent high basal levels and further increase following burning. In the absence of convincing evidence to the contrary, it does not seem likely that the pituitary ACTH release following burns in dogs with isolated pituitaries is due to sympathetic or parasympathetic nervous connections to the anterior pituitary.

Another possibility is that the pituitary has become more sensitive to peripherally produced humoral agents which are ineffective in the normal animal. Experiments with the isolated leg preparation published elsewhere (17) give strong support for the concept that pituitary ACTH release following traumatic stimuli, such as burns or operative trauma, is totally dependent upon nervous impulses to the brain, and involves no humoral component from the peripheral tissues. That the isolated pituitary preparation is no more sensitive to such hypothetical but unproven "wound hormone" (24) than the normal animal is shown by experiment B-100 in which an adrenal cortical response was observed following burn to the innervated but not the denervated leg.

According to the hypothesis set forth by Sayers (25), the pituitary release of ACTH is under the control of the peripheral level of blood 17-hydroxycorticosteroids, in addition to other factors. Table 3 shows that decreased peripheral blood levels do not account for pituitary ACTH release, since peripheral blood levels and adrenal venous corticosteroid outputs were simultaneously elevated 24 hours following brain removal. It is apparent that the pituitary release of ACTH occurs in the presence of high peripheral blood levels of corticosteroids. Preliminary studies do not demonstrate blood pH or electrolyte changes which might be conjectured as pituitary-stimulating factors in dogs with brains removed.

The most likely explanation for the release of ACTH following trauma in dogs with isolated pituitaries is the production of an ACTH-releasing neurohumor by the hind brain, spinal cord, or elsewhere. Previous studies have shown that spinal cord section at C7 completely prevents ACTH release following burns or operative trauma (17). Accordingly it would

seem likely that nervous impulses pass from the burned extremity up the spinal cord to an area located somewhere between the medulla and inferior colliculus and that the hind brain produces an ACTH-releasing neurohumor which passes by the systemic blood stream to the pituitary. We do not have evidence upon which to base a choice among the cerebellum, pons, and medulla for this center. The median eminence of the hypothalamus does not need to be present in dogs with isolated pituitaries, either to maintain high resting corticosteroid levels or for the further increase in corticosteroid output which occurs following burns. These responses occurred in several animals with no median eminence present as determined by serial sections. Accordingly, it seems most likely that a neurohumor is produced by the hind brain (HBF—hind brain factor) and passes by the systemic blood stream directly to the pituitary, effecting ACTH release.

Although the evidence points strongly toward production by the hind brain of an ACTH-releasing neurohumor, another possibility must be considered. Spinal cord section studies have not yet been performed in dogs with isolated pituitaries, and it is possible that the spinal cord itself produces a substance which has the ability to release ACTH after removal of the higher brain centers. In addition it is unlikely, but conceivable, that a substance which releases ACTH is produced elsewhere than in the central nervous system. Guillemin (26) has found *in vitro* ACTH-releasing potency not only for hypothalamic tissue, but also for a substance present in intestinal musculature. It is possible that the production of this substance depends upon a spinal reflex involving a peripheral afferent nerve, hind brain or spinal cord, and efferent to the intestine. Although unlikely, such a possibility is not ruled out by our data. In addition it is established that a similar reflex mechanism results in epinephrine release from the adrenal medulla and it is possible that epinephrine itself is an ACTH-releasing agent in dogs with isolated pituitaries. Epinephrine is ineffective as an ACTH-releasing agent in the intact dog (27) but nothing is known concerning its action in animals lacking higher brain centers. The fact that several animals in Tables 1 and 2 did not show a significant adrenal cortical response following burns, although a highly significant catechol amine response did occur, seems to be somewhat against the hypothesis that epinephrine is the ACTH-releasing agent.

Mason has stimulated various areas of the brain in conscious monkeys and determined the peripheral blood 17-hydroxycorticosteroids. He found hippocampal stimulation to be inhibitory and hypothalamic (28) and amygdaloid (5) stimulation to result in elevated blood corticosteroid levels. Martin and co-workers (6) found that removal of the amygdaloid nuclei in cats resulted in a marked increase in adrenal cortical secretion, associated with a reduction in general activity and resistance to emotional stimuli. These findings indicate a complex interplay of excitatory and inhibitory factors in intact animals. It is probable that the usual mecha-

nism of adrenal cortical activation in the intact animal depends upon hypothalamic stimulation by impulses passing up the spinal cord and through the reticular formation. The hypothalamic neurohumor passes by the hypophyseal portal system or through the systemic blood stream to the pituitary resulting in ACTH release. Although inhibition of the hypothalamus, as well as of the lower ACTH-releasing center, in the resting state probably accounts for the basal corticosteroid outputs in either anesthetized or unanesthetized normal dogs, this inhibition can be broken through by stimulatory impulses from below. Hypothalamic lesions abolish reactivity (29) because HBF is inhibited by impulses of a nervous or humoral nature from higher central nervous system centers. After brain removal, HBF inhibition is removed, with resulting high basal corticosteroid outputs. Trauma to the extremity results in a further increase in production of HBF, and corresponding increased ACTH output.

The situation with respect to the effect of brain removal on adrenal medullary secretion both in the resting stage and in response to trauma seems much more clear-cut. As far as can be determined, brain removal down to the level of the pons, either with or without hypophysectomy does not alter adrenal medullary response from normal. This is not to imply that emotional stimuli such as fear, which leads to impulses which pass from the cerebral cortex down to the lower centers and results in adrenal medullary secretion, are present in dogs with brain removal. The present discussion, however, is limited to stimuli such as burns or operative trauma. There is an abundance of evidence suggesting that the cerebral cortex exerts a tonic or inhibiting influence on lower central nervous system centers which control adrenal medullary secretion (30, 31, 32, 33). Dogs with isolated pituitaries have normally low basal outputs of adrenal medullary catechol-amines, and this would seem to rule out the possibility of a tonic inhibition of the adrenal medullary secretion center from higher areas.

A previous series of experiments in our laboratory has demonstrated that spinal cord section at C7 abolishes the adrenal venous catecholamine output increase following burns or operative trauma (34). Accordingly, the finding that dogs with brains removed have normal adrenal medullary responses following burns or operative trauma makes it seem likely that the center for control of adrenal medullary secretion is located between C7 and the pons. This work represents confirmation of the theories of Cannon (3) and Bard (35) who demonstrated that transection of the brain above the level of the medulla left intact the adrenal medullary response to trauma, but that spinal cord section below the medulla left an animal which did not give an adrenal medullary response following trauma. Additional points added to this theory by the present studies are that there is no tonic inhibition of the adrenal medullary activation center located in the medulla oblongata, and that humoral participation by central nervous

centers above the medulla is not needed for maximal adrenal medullary response following trauma.

There is nothing in this work which implies that impulses emanating from higher centers cannot activate the medullary center for catecholamine output under certain circumstances. The studies of Hess (4), Von Euler and Folkow (36), and Hoff and co-workers (37) emphasize that stimulation of various areas in the central nervous system results in increased adrenal medullary secretion. The emphasis of the present studies is that these centers are not essential to the marked adrenal medullary catecholamine response which occurs following trauma.

CONCLUSIONS

Dogs with isolated pituitaries have high resting adrenal venous corticosteroid outputs, and about two-thirds of such animals respond to burn trauma with a further increase. This is interpreted to mean:

a. Higher areas of the brain tonically inhibit lower areas which produce and release ACTH-releasing neurohumors in the dog.

b. An area which produces an ACTH-releasing neurohumor is located in the hind brain. This hind brain factor (HBF) acts through the systemic blood stream to effect ACTH release from the pituitary, or possibly, in the intact animal to stimulate release of the hypothalamic ACTH-releasing neurohumor. HBF is inhibited by nervous or humoral mechanisms emanating from the higher central nervous system. The data of this report do not rule out other possible explanations for the ACTH-releasing effect of trauma in dogs with isolated pituitaries.

The center essential for adrenal medullary activation following burns or peripheral nerve stimulation is located in the medulla oblongata. There is no evidence for tonic inhibition of this area by higher CNS structures.

Acknowledgment

The technical assistance of David Droppleman is gratefully acknowledged.

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FURTHER STUDIES ON THE REGULATION OF PITUITARY ACTH IN RATS WITH HYPOTHALAMIC LESIONS[1]

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ABSTRACT

"Acute" or "chronic" lesions in the median eminence can block the adrenal ascorbic acid depletion normally occurring one or four hours following unilateral adrenalectomy. Such lesions can induce adrenal atrophy in the presence of normal sized testes. Adrenal ascorbic acid depletion in rats with adrenal atrophy is variable, and some of them do not have diabetes insipidus. Pituitary ACTH content is within normal limits in rats with "acute" lesions but falls to 50% of normal in animals with "chronic" lesions.

RELEASE of adrenocorticotrophin (ACTH) following stress is deficient in rats with hypothalamic lesions destroying the median eminence of the tuber cinereum (1–4). These animals exhibit a complete absence of response to acute noxious stimuli but show diminished responses to chronic stimuli (5). The present report presents experiments designed to delineate further the degree of blockade in the pituitary-adrenal system present in rats with hypothalamic lesions.

METHODS

Hypothalamic lesions were made in the median eminence of male, Wistar rats using the Krieg stereotaxic instrument (1, 2). Two types of rats with lesions were used for experiments. Those which had been operated upon 48 hours previously are designated rats with "acute" lesions, while those which were used 3 or more weeks after operation are called rats with "chronic" lesions. Unless otherwise specified, the animals with "acute" lesions drank at least 100 ml. of water during the first 24 hours post-operatively, and those with "chronic" lesions drank 200 ml. or more per day; such rats do not respond to acute stress (6).

Adrenal ascorbic acid depletion was determined in response to the stress of unilateral adrenalectomy under ether anesthesia using methods previously reported (1).

Pituitaries were removed from rats immediately after sacrifice, ground in 0.5 ml. of 0.1N HCl per anterior lobe and stored in the deep freeze. Pools of six or more glands were suitably diluted and assayed at 2 or more dose levels in hypophysectomized rats[2] by the

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[2] Hypophysectomized rats were obtained from Hormone Assay Laboratories, Chicago.

TABLE 1. ADRENAL ASCORBIC ACID DEPLETION FOUR HOURS FOLLOWING UNILATERAL ADRENALECTOMY IN RATS WITH LESIONS

Type of animal	Adrenal ascorbic acid depletion (mg./100 gm. adrenal)
Normal	190 ± 7 (6)[1]
"Acute" Lesions	22 ± 11 (6)
"Chronic" Lesions	54 ± 33 (6)

[1] Mean ± S.E.M.; the number inside the parenthesis is the number of rats in the group.

method of Sayers *et al.* (7). Ten or more rats were used for assay of each pool of pituitaries, and the results compared to those obtained with U.S.P. reference standard corticotrophin.[3]

RESULTS AND DISCUSSION

Previous results indicated that the depletion in adrenal ascorbic acid which occurs one hour after unilateral adrenalectomy is blocked in rats with "acute" or "chronic" lesions in the median eminence (1, 2, 4). In the present experiments, the second adrenal was removed 4 hours post-unilateral adrenalectomy since some workers have presented evidence for a delayed metabolic phase in ACTH release (8, 9). The results (Table 1) indicate that the ascorbic acid depletion seen in normals 4 hours after unilateral adrenalectomy failed to occur in rats with "acute" lesions. There was no significant response in the group of rats with "chronic" lesions; however, the ascorbic acid response was quite variable. Two rats showed an increase in ascorbic acid of the second adrenal; two showed a small drop; and two gave responses within the normal range. It would appear that hypothalamic lesions can prevent the ascorbic acid depletion occurring 4 hours after unilateral adrenalectomy.

Adrenal atrophy can follow lesions in the median eminence (10). The results shown in Table 2 demonstrate that this adrenal atrophy can occur in the presence of normal sized testes, suggesting that adrenal atrophy is a

TABLE 2. RATS WITH LESIONS AND ADRENAL ATROPHY BUT WITH NORMAL TESTES[1]

Type of animal	Adrenal wt. (mg.)	Adrenal ascorbic acid depletion (mg./100 gm. adrenal)	Water intake (ml./day)	Testes wt. (gm.)
Normal	35 ± .7 (53)	165 ± 10 (10)	37 ± 2 (8)	3.0 ± .1 (10)
Lesions; normal ascorbic acid depletion	21 ± .7 (13)	190 ± 14 (13)	52 ± 1 (13)	2.9 ± .1 (13)
Lesions; no ascorbic acid depletion	19 ± .8 (8)	5 ± 11 (8)	153 ± 31 (8)	3.0 ± .1 (8)

[1] Adrenal atrophy was considered to be present if the adrenal weight was more than tw standard deviations smaller than that of controls. The same criteria were applied to defin testicular atrophy.

[3] Kindly supplied by Dr. Herman Cohen of Princeton Laboratories.

specific result of the lesions and not secondary to a general depression of all anterior lobe function. Since a smaller pituitary remnant is required to maintain adrenal weight than is required to maintain gonadal function (11), the possibility that the adrenal atrophy is due to vascular damage to the pituitary consequent to injury to the hypophysial portal vessels appears to be unlikely.

A correlation between adrenal atrophy and diabetes insipidus has been observed (10). In the present series of rats with adrenal atrophy, diabetes insipidus was generally present; however, six animals were encountered with adrenal atrophy and normal water turnovers.

Some rats with atrophic adrenals responded to unilateral adrenalectomy with ascorbic acid depletion, whereas others did not. In general, those rats which failed to exhibit ascorbic acid depletion had more severe dia-

TABLE 3. PITUITARY ACTH IN RATS WITH HYPOTHALAMIC LESIONS

Type of rat	Pituitary ACTH content		Pituitary weight[1]	
	Mu.	% of normal	Mg.	% of normal
Normal	107 (68–168)[2]	100	7.6 ± .5 (6)[3]	100
Unilateral adrenalectomy	121 (78–188)	113		
"Acute" Lesions	78 (52–119)	73		
"Chronic" Lesions	51 (34–76)[4]	48	6.3 ± .3 (14)[5]	84

[1] Anterior Lobe only.
[2] Figures in parenthesis are 95% confidence limits.
[3] Mean ±S.E.M.; figures in parenthesis are the number of pituitaries weighed.
[4] Significantly less than the value for any other type of rat.
[5] Significantly less than normal ($P < 0.05$).

betes insipidus than those which responded with depletion. All of the rats failing to exhibit ascorbic acid depletion had diabetes insipidus as reported previously (10). Apparently basal secretion rates of ACTH can be considerably reduced, as evidenced by adrenal atrophy, and yet ACTH secretion in response to stress may still occur.

As reported previously, control adrenal ascorbic acid concentrations were elevated above normal in rats with either acute or chronic lesions (1, 6). Animals with acute lesions had either gained or lost small amounts of body weight since operation whereas those with chronic lesions had invariably gained weight some of them even becoming obese (1, 6).

Pituitary ACTH was determined in rats with "acute" and "chronic" lesions, and the values were compared to those obtained in normal rats and those subjected to unilateral adrenalectomy one hour earlier (Table 3). The latter control group was included, because all rats with lesions had been subjected to unilateral adrenalectomy one hour prior to sacrifice in order to evaluate the ascorbic acid depletion from this stress. The rats with lesions chosen for this ACTH assay had all failed to show a significant ascorbic acid depletion one hour after adrenalectomy.

Pituitary ACTH was not significantly altered by unilateral adrenalec-

tomy in the control rats and was not significantly lowered by "acute" hypothalamic lesions; however, the value found in rats with "chronic" lesions was only 48% of the control and was significantly lower than that found in any of the other groups of animals. The weight of the anterior lobe in rats with "chronic" lesions was also slightly less than normal, and adrenal weights of this group of animals were subnormal.

Pituitary ACTH content reflects a balance between synthesis, release, and possible destruction of ACTH in situ (12). There is no information available on this latter possible cause of decreased pituitary ACTH content in rats with lesions. Since these hypothalamic lesions reduce the output of ACTH in response to stress and also appear to decrease basal secretion of ACTH, as evidenced by the adrenal atrophy seen here, the decrease in pituitary ACTH is interpreted to mean a reduced rate of synthesis of ACTH in rats with lesions.

It is of interest to compare the results obtained in the present experiment with those of a previous study (2) (Table 4), in which pituitary ACTH from adrenalectomized rats and adrenalectomized rats with lesions was determined. It can be seen that, although pituitary ACTH is approxi-

TABLE 4. SUMMARY OF DATA ON ACTH CONTENT OF PITUITARIES OF RATS WITH MEDIAN EMINENCE LESIONS

Normal	Lesions
100 ± 23%	48 ± 11%
Adrenalectomized[1]	Adrenalectomized with Lesions[1]
200 ± 25%	100 ± 15%

[1] Data of McCann and Sydnor (2).

mately 50% of normal in rats with lesions, two weeks following adrenalectomy it rises to a value equal to that of normal rats. The content in the adrenalectomized rat with lesions is still only one half of that found in adrenalectomized animals without lesions where the ACTH content has risen to 200% of control. Thus, it would appear that the pituitary of the rat with lesions is able to respond to the decrease in blood corticoids resulting from adrenalectomy with an augmented synthesis of ACTH. Assuming that these lesions completely eliminate neural control of the pituitary, another mechanism of control must account for this residual effect. In general, then, the "denervated" pituitary synthesizes and secretes ACTH at a lowered basal rate and is unable to respond to acute stimuli with augmented ACTH discharge, but it retains at least a partial ability to respond to chronic influences, such as adrenalectomy, with augmented synthesis and release of ACTH (5).

Acknowledgment

The technical assistance of A. Fruit is gratefully acknowledged.

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HISTOLOGICAL CHANGES IN THE ORGANS AND TISSUES OF MIGRATING AND SPAWNING PACIFIC SALMON (GENUS *ONCORHYNCHUS*)[1]

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ABSTRACT

A histological study was made of the various organs and tissues of the Pacific salmon at successive stages of sexual maturation from the sea to the spawning grounds. Pronounced changes were found in the body structures of the spawning salmon prior to death. These consisted principally of degeneration with or without atrophy as shown by the stomach, liver, spleen, thymus, thyroid, gonads, pituitary, kidney and cardiovascular system. On the contrary, certain organs exhibited hypertrophy and hyperplasia, e.g., adrenal, pancreatic islets and skin. The inception of some of the above changes was detected several months before full sexual maturity. Comparison with the histological alterations occurring in Cushing's syndrome, experimental hyperadrenocorticism and aging, suggested that many of the tissue changes found in the salmon could be ascribed to the effects of hyperactivity of the adrenal gland. Previous finding of high concentrations of 17-OHCS in the plasma of spawning salmon lends support to this inference.

AMONG the many unusual features of the life cycle of Pacific salmon is the fact that they all appear to die of the same cause—universal death following the first spawning can be interpreted scarcely in any other way. Onset of the lethal process is frequently evident before spawning. Many Pacific salmon at full sexual maturity exhibit obvious degenerative changes, fungus infection, focal necrosis of the skin, loss of muscular strength and balance, but others reach this stage in apparently good condition. Following spawning, deterioration progresses rapidly, all fish are affected, and death ensues within a few days to two weeks. Some sexually ripe salmon die before spawning. That profound alterations are occurring in the tissues at this terminal period of the salmon's life can hardly be doubted. Indeed striking degenerative changes in certain organs were described by Greene (1) in 1926. But since his histological studies were limited in scope and did not include a number of body structures, a systematic microscopical examination of the salmon at successive stages in its sexual cycle seemed to be indicated.

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The present communication constitutes a continuation of our inquiry into the nature of the post-spawning death of the Pacific salmon. In previous papers, histological changes in the pituitary gland and the adrenal cortical tissue were described (2, 3, 4). Both glands were found to exhibit markedly increased activity (cellular proliferation and hypertrophy) during gonad maturation, followed at full sexual maturity by degeneration, more pronounced and constant in the pituitary than in the adrenal. The latter was characterized by extensive hyperplasia. Study of the other tissues and organs of the spawning salmon has revealed striking alterations in structure and cytology. These changes ranged from hypertrophy and hyperplasia on the one hand to atrophy and degeneration on the other.

MATERIALS AND METHODS

Sexually immature king salmon (*O. tschawytscha*) with infantile gonads were caught in the sea in Monterey Bay and in the open ocean just off Bolinas Bay. Those with gonads in varying stages of sexual maturation were secured at several stations on the Sacramento River. Sexually mature fish were taken at the spawning grounds of the same river.[2] Maturing and ripe blueback salmon (*O. nerka nerka*) were obtained at Entiat on the Columbia River.[3] Spawning kokanee (*O. nerka kennerlyi*) were caught in Donner Lake, California while immature kokanee were provided by the California Department of Fish and Game at their Lake Tahoe hatchery. A total of 104 king salmon, 6 blueback, and 12 kokanee were studied.[4]

All skin specimens were taken from the same general area just cephalad and lateral to the dorsal fin. Kidney specimens were taken from the mid-portion of the kidney. Histological methods used were described in a preceding publication (5). Additional special stains for certain tissues were employed; Heidenhain iron hematoxylin to bring out granules in glandular and granulosa cells of the stomach and intestine, iron hematoxylin azan for muscle fibers, Masson trichrome and Mallory technique for collagenous tissues, Gomori aldehyde fuchsin stain counterstained with Masson trichrome as employed by Abrams *et al.* (6) to bring out distinctive differences between the alpha and beta cells in the islets of Langerhans. Fat stains used were Sudan black B, Sudan orange and Oil Red 0.[5]

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[2] To the following members of the California Department of Fish and Game we wish to express our sincere appreciation for their generous assistance in securing material for this investigation. Dr. Alex Calhoun, Mr. Leo Shapovalov, Mr. Jack C. Fraser, Mr. Allen F. Pollitt, Mr. Richard Hallock, Mr. William Van Woert, Mr. James A. Hinze, Mr. Donald P. Evins, Mr. Phillip Murray, Mr. John Riggs, and Mr. T. James Ready. We are grateful to Mr. John Pelnar, Coleman Station, U. S. Fish and Wildlife Service at Anderson, California, for his cooperation on repeated occasions in making available to us specimens of ripe and spent salmon.

[3] We wish to thank Mr. Roger E. Burrows, Director, Salmon Cultural Station, U. S. Fish and Wildlife Service, Entiat, Washington, for his kindness in providing a number of specimens of blueback salmon for this study.

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RESULTS

Skin

The skin of the salmon resembles in general that of other fishes (7). Figure 1, skin of an immature sea salmon, shows a thin epidermal layer below which is an open reticular zone in which the scales lie. Next a thick laminated stratum compactum rests on fatty tissue and muscle. With sexual maturation the skin becomes much thicker (Fig. 2). This change involves all three principal zones. The epithelium shows very active proliferation of the germinal layer, characterized by tall columnar cells (Fig. 4) as compared with the much smaller germinal cells of the immature fish (Fig. 3). The reticular zone becomes solidified. The scales are deeply buried below the epithelium[6] and a 2 to 3 fold thickening of the stratum compactum takes place.

Comparative measurements of the skin of immature and spawning king salmon showed an average thickness of 420 μ for the former and 1370 μ for the latter. This difference might be accounted for partly by the larger size of the spawning salmon, however, immature and mature kokanee of similar size were found to have skin thicknesses of 213 μ and 816 μ respectively. The skins of male fish averaged about 25% thicker than those of the females. Increasing thickness of the skin appears to begin several months before spawning.

At the same time that active growth of the skin epithelium is occurring, degeneration of the scales is taking place. So-called scale absorption (Figs. 5 and 6) consists first in disappearance of peripheral circuli then loss of scale substance. Scales of females showed less marked absorption than did those of the males.

Stomach and Intestine

Greene (8) has described the anatomy and histology of the stomach and intestine of the normal immature king salmon and has reported on their marked atrophy in the spawning fish (1). Since no one, as far as we can find, has pictured these changes, we present the accompanying photographs which illustrate the most conspicuous alterations. In the spawning salmon the stomach is greatly contracted. Figures 7 and 8 show the difference in diameter of the empty stomach of a feeding sea salmon and a fully mature fish of similar size. Figures 9 and 10 reveal in more detail the marked changes that have taken place by the time of spawning. The villi have disappeared leaving smooth-surfaced folds of the mucosa. The tall epithelial cells of the sea salmon (Fig. 11) tend to become low columnar and in certain instances have shrunken to an almost cuboidal shape (Fig.

[6] It is almost impossible to secure scales from sexually mature salmon by scraping with a knife. We have found that a strip of skin placed in 2 per cent KOH for 48 to 72 hours becomes so digested that the scales are easily detached.

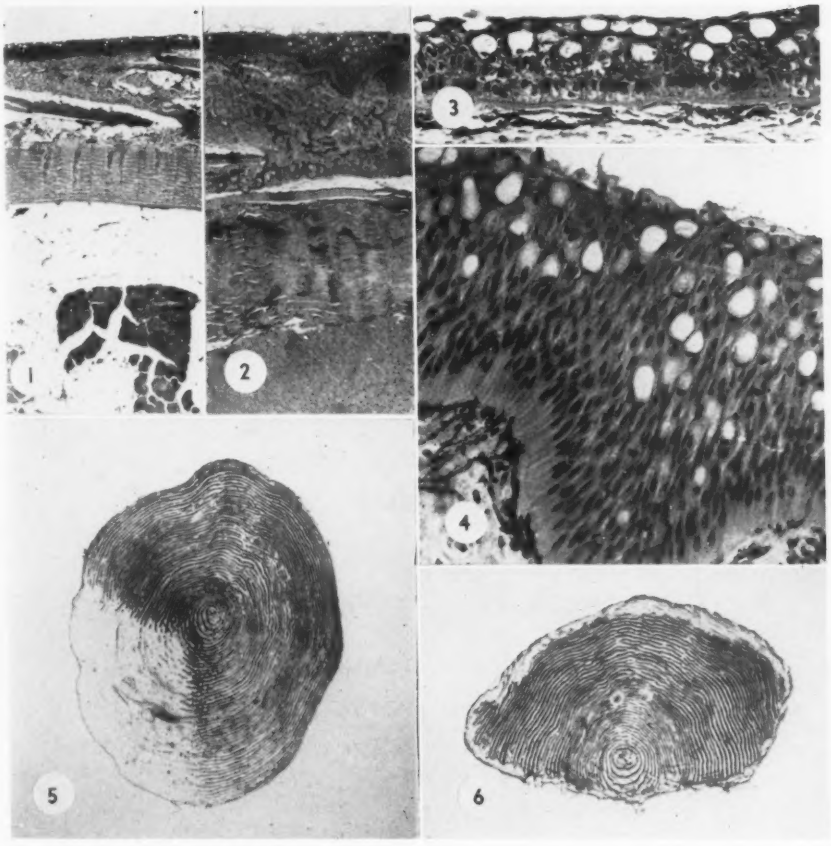

FIG. 1. Skin of immature male king salmon taken on spawning migration—length 30.5 inches. Scales are seen in the loose areolar tissue of the dermis. H. and E. ×40.

FIG. 2. Skin of spawning male king salmon—length 24 inches. H. and E. ×40

FIG. 3. Epidermis of immature salmon shown in Fig. 1. Cells of germinal layer are low columnar. H. and E. ×225.

FIG. 4. Epidermis of spawning salmon shown in Fig. 2. Shows marked proliferation. Germinal cells high columnar. H. and E. ×225.

FIG. 5. Scale of an immature kokanee. 4 years of age. Unstained ×13.5.

FIG. 6. Scale of spawning kokanee, showing marked absorption. Lower caudal $\frac{1}{3}$ of scale has been lost and peripheral circuli of remaining portion are disappearing. Unstained ×16.

12). While the number of epithelial cells has shown a very great diminution they are still intact. The stratum compactum, a dense narrow band of collagenous tissue which lies near the outer margin of the mucosa is seen as a thickened convoluted structure (Fig. 10). The heavy circular muscle layer in the spawning salmon exhibited marked atrophy and in places fragmen-

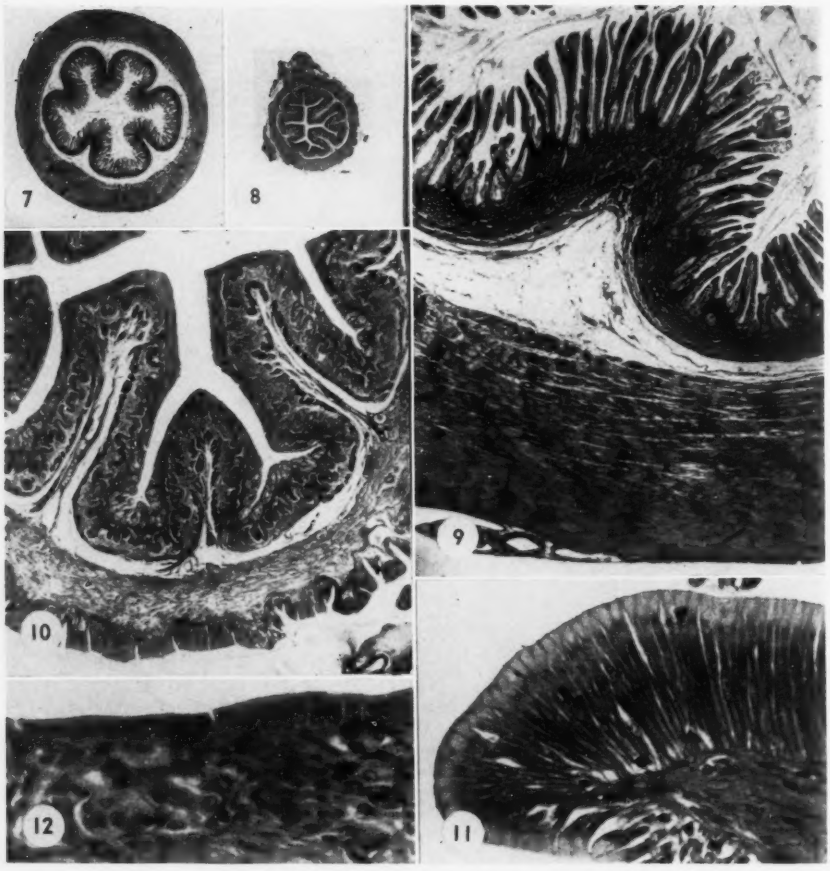

FIG. 7. Cross section of stomach of an immature, sea, male king salmon. 26 inches in length in 4th year of age. Iron, hematoxylin, azan, ×2.5.

FIG. 8. Cross section of stomach of spawning female king salmon 24 inches long, in 4th year of age. Iron, hematoxylin, azan, ×2.5.

FIG. 9. Higher magnification of an area of stomach of sea salmon shown in Fig. 7. Note numerous tall villi and thick muscle layer. Iron, hematoxylin, azan, ×22.

FIG. 10. Higher magnification of stomach of spawning salmon shown in Fig. 8. Note loss of villi and thinning of muscle coat. Iron, hematoxylin, azan, ×22.

FIG. 11. Epithelial cells lining villi in stomach of sea salmon shown in Fig. 8. H. and E. ×400.

FIG. 12. Epithelial cell layer covering surface of mucosal folds shown in Fig. 9. H. and E. ×400.

tation of the muscle fibers. Comparative measurements of this muscle layer in sea and sexually mature fish gave an average thickness of 1510 μ for the former and 344 μ for the latter. The external or longitudinal muscle, scarcely visible in Figure 9, has become a much thickened zone in the

spawning fish (Fig. 10) marked by rugae. Its composition is largely hypertrophied plexuses of Auerbach, blood vessels and newly-deposited collagenous tissue. Scattered individual muscle fibers are seen, some of them degenerating, but it is difficult to ascertain whether or not they are diminished in number.

These changes in the stomach of the migrating salmon begin some time before spawning. While marked contraction accompanying starvation is always present from the time the salmon leaves the sea, significant atrophy has not been observed until 2–3 months before full sexual maturity. During the mid-stage of migration the eosinophilic granular cells of the mucosa are present in greater abundance than previously and are heavily granulated.[7] At spawning these cells are present in even greater numbers but are less heavily granulated. Similar degranulation is characteristic of the cells of the gastric glands.

The changes occurring in the intestine and pyloric cecae of the spawning salmon are in general like those of the stomach. In addition, degeneration and desquamation of the epithelium was found in some fishes, the mucosa being replaced by a dense mass of cells, polymorphonuclear, mononuclear and multinucleated giant cells.

Liver

The principal differences between the livers of fishes (including salmonids) and mammals is, (1) the absence of any definite lobulation in the former and, (2) two-layered cords of cells instead of a single layer characteristic of the mammalian liver (12). During transition from the sea to the spawning grounds the liver of the salmon undergoes marked changes. Before the beginning of the spawning migration the liver cells accumulate a large store of fat (Fig. 14). Prior to this time the liver shows little or no fat deposition as judged by staining methods (Fig. 13).[8] The amount of fat in the liver diminishes gradually as the fish ascends the river and by the time of spawning it can no longer be detected by fat stains.[9] The first evidence of degenerative change in this organ was found in salmon with well-developed gonads, circa 6 weeks to 2 months before spawning. This consisted chiefly of alterations in the nuclei which were irregular in shape and size; the chromatin was coarse and consisted of granules rather than

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[7] Greene (9) believes that these cells may be a source of lipase. Bolton (10) considers the granulosa cells to be mast cells. However, Weinreb and Bilstead's (11) histochemical studies of these cells indicated that they were not mast cells.

[8] Fat storage would appear to depend largely on the kind of food taken. Just before migration, salmon feed actively on fat-rich pilchards and anchovies. The varying kinds of food found in the stomachs of salmon off the California coast during different seasons of the year has been reported by Merkel (13).

[9] This may not be true of salmon spawning in short coastal streams. We know of no studies on the fat content of the livers of such fishes.

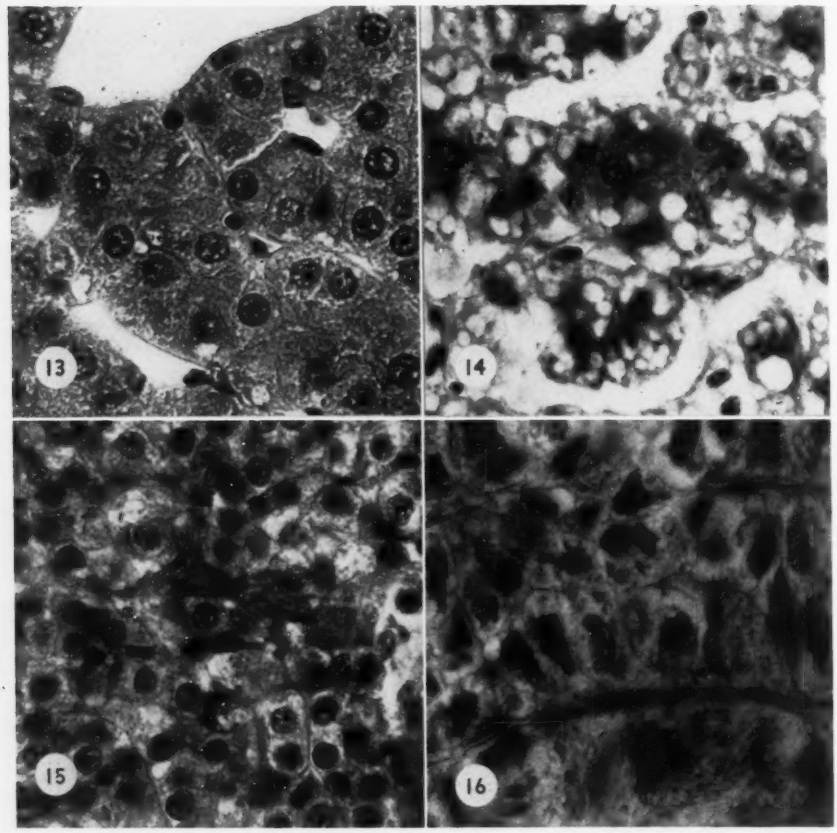

FIG. 13. Liver of sexually immature sea king salmon. H. and E. ×900.

FIG. 14. Liver of sexually immature king salmon at beginning of spawning migration. Liver cells loaded with fat droplets of varying size. H. and E. ×900. Presence of fat corroborated by stain with Sudan orange.

FIG. 15. Liver of male king salmon with well-developed testes. Would probably spawn within one to two months. Shows beginning degeneration of cells. H. and E. ×900.

FIG. 16. Liver of spent kokanee taken from Donner Lake. Marked generalized degeneration of hepatic cells. H. and E. ×900.

threads. The size of the nuclei was unchanged. However the cells had become somewhat smaller giving the appearance of a greater number of nuclei per unit area of liver substance. In addition the cytoplasm of many cells exhibited a moth-eaten aspect and here and there nuclei were disintegrating (Fig. 15).

With full sexual maturity and spawning cellular degeneration had become pronounced. The amount of cytoplasm was diminished and what remained was irregularly distributed in small masses giving the cell a

curdled appearance. The nuclear changes varied from the beginning disturbances described above to intense pycnosis, fading of nuclear outlines and complete dissolution (Fig. 16). Considerable variation in the intensity of the degenerative changes was observed in individual fish. The most constant marked liver deterioration was found in kokanee. No changes in the supporting tissues of the liver were observed.

Kidney

The structure of the kidney of salmonid fishes resembles that of the mammal except for the absence of loops of Henle and the presence of a large number of lymphoid cells in the parenchyma. Beginning evidence of degenerative change in the kidney was found in fourth year sea salmon with gonads still very small and undeveloped. This consisted of incipient but definite glomerular capillary sclerosis and some thickening of Bowman's capsule. Second and third year fish did not show these changes. With progression of the migration and maturation of the gonads the changes were more frequently seen—in third year as well as fourth year fish. Fully mature and spawning salmon (including several second year fish—grilse) all showed kidney degeneration. Glomerulo-capillary sclerosis was often pronounced, frequently obliterating the lumen of the capillaries (cf. Figs. 17 and 18). This was usually associated with a much thickened Bowman's capsule. With progression of the process the glomerular tuft was transformed into a mass of hyaline-like material. Occasionally a very large fibrosed glomerulus was observed. In some fish the glomeruli were greatly dilated, edematous, and the capillary tuft shrunken (Fig. 19).

Most spawning fish showed relatively mild deterioration of the tubules consisting of vacuolization of the lining cells, some loss of cytoplasm and occasional disappearance of nuclei. In some instances more marked tubular degeneration was present (Fig. 20). This was focal in distribution. While the tubules were often filled with finely granular material there was no suggestion of casts. Scattered patches of edema and hemorrhage were found in several fish. The number of lymphoid cells in the kidney parenchyma was considerably diminished in the spawning salmon.

Spleen

The spleen of teleost fishes differs from that of mammals principally in two respects; first, there are no germinal centers, the lymphoid tissue is diffuse and intimately mingled with the red pulp (14); and second, the connective tissue framework is much less evident. Beginning change in spleen histology, as indicated by increase in the connective tissue stroma, was found in migrating salmon while the gonads were still in the early stages of development. With progressive gonad maturation, diminution in the number of lymphoid cells and further increase in fibrous tissue occurred. At full sexual maturity the lymphocytes showed marked depletion to com-

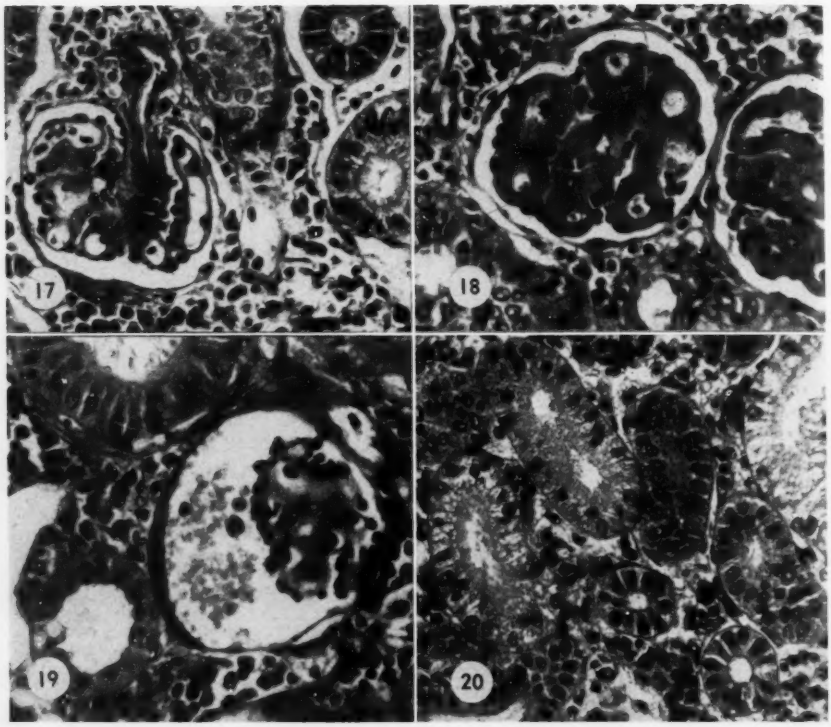

FIG. 17. Kidney of immature sea king salmon showing normal glomerulus. H. and E. ×400.

FIG. 18. Kidney of migrating king salmon with developing ovaries, exhibits marked capillary glomerulosclerosis. H. and E. ×400.

FIG. 19. Kidney of spawning king salmon grilse shows marked edema and compression of glomerular tuft. H. and E. ×400.

FIG. 20. Kidney of spawning king salmon showing degeneration of tubules. H. and E. ×400.

plete disappearance; there was an increase in the number of large mononuclear cells, free and fixed and newly-formed connective tissue occupied a considerable portion of the spleen substance (Figs. 21 and 22). Necrosis was found in only one out of 16 spleens of spawning fish and this was focal in distribution. No particular difference was noted in the amount of blood in the spleen between sea and spawning salmon. Pigment granules were not observed in fish with immature gonads, and were infrequent in salmon nearing spawning. In ripe and spawning fish they were present constantly but few in number.

Thymus

While the thymus in fish and mammals has a common origin in the wall of the brancheal clefts, in the former it remains an integral part of the

epithelium where it can be seen as an opaque white bean-shaped structure located at the angle of the operculum and the dorsal body musculature. The histological features have been described by Deansley for the trout (15) and by Hafter for *Astyanax* (16). This tissue in the immature salmon consists of a superficial zone composed of 1–2 layers of flattened epithelial cells and 2–4 layers of large mucus-secreting cells which cover a densely packed mass of small thymocytes (Fig. 23). A thick connective tissue capsule separates the thymus from the muscle. In the thymic tissue there occurs an occasional accumulation of light-staining cells with relatively large vesicular nuclei, arranged concentrically about a lumen which occasionally

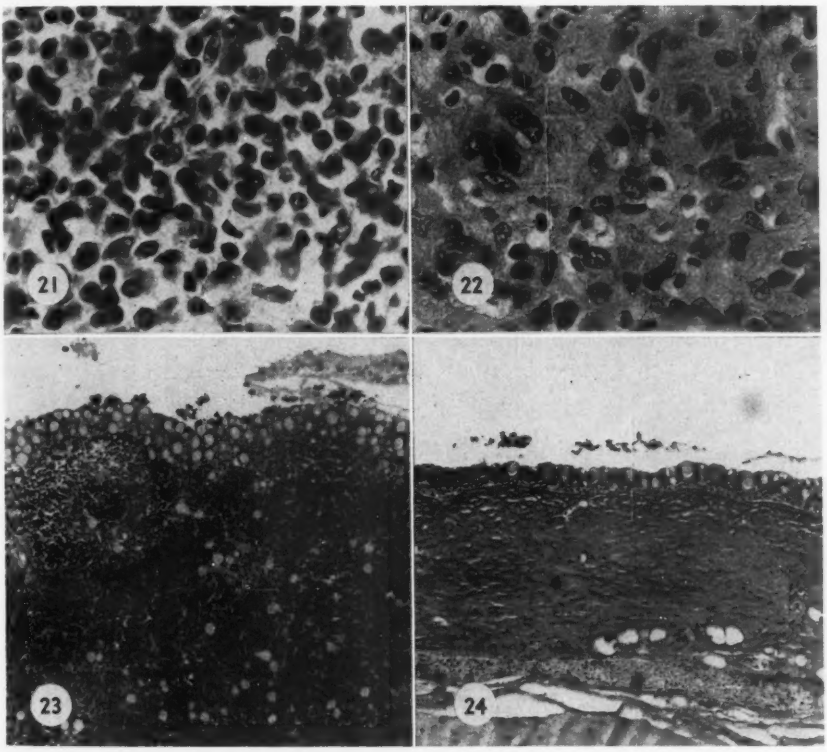

FIG. 21. Spleen of immature sea king salmon. Pulp composed chiefly of small lymphocytes, very little fibrous tissue. H. and E. ×750.

FIG. 22. Spleen of spawning king salmon. Small lymphocytes rare, many large nuclei, fibrous tissue abundant. H. and E. ×750.

FIG. 23. Thymus of immature sea king salmon in 4th year of age. Densely packed layer of thymocytes. H. and E. ×110.

FIG. 24. Thymus of spawning king salmon grilse in 2nd year of age. Thymic tissue has shrunk to a narrow superficial layer. Thymocytes have practically disappeared. H. and E. ×100.

contained amorphous material. Similar structures have been described in the mammalian thymus (17). Hassel's corpuscles were not detected. This corresponds with Deansley's and Hafter's findings.

Involution of the thymus was observed in one fourth year sea salmon. Two others of the same age, however, showed a thick layer of thymocytes as did the younger fish. Most of the migrating fish, including second and third year salmon exhibited a pronounced diminution in the number of thymocytes, which were replaced in part by mononuclear cells and an increased number of goblet cells. This resulted in reduced thickness of the gland. Whenever involution was occurring a single row of cuboidal or low columnar cells, contiguous with the basement membrane, was present.

Marked thymic involution was found in all 16 spawning salmon examined including four second year fish (grilse) (Fig. 24). Thymocytes were often rare or even apparently absent. Measurements of the thickness of the thymus gave averages of 427 μ for the sea salmon, 144 μ for the migrating and 82 μ for the spawning fish. By the time of spawning the row of cells on the basement layer, noted above, had taken on the appearance of fibroblasts.

These observations suggest that involution of the thymus in the salmon is associated both with aging and the process of sexual maturation.

Pancreas

As in most other teleost fishes the pancreas of the salmon is a diffuse gland present in fragments situated principally between the pyloric cecae. The islets of Langerhans are found in the exocrine or zymogenous tissue. In the sexually immature sea salmon with infantile gonads the islets are small and very few in number (Fig. 25). Fat in considerable quantities was present in the pancreas of some of the sea fish examined, absent in others. This difference apparently depends on the time of year and the kind of food being eaten as indicated in discussing the presence of stainable fat in the liver.

Increase in the number and size of the islets occurred on the spawning migration. Whether this paralleled the degree of gonad maturation is not clear but hypertrophy of the islets was more marked in those fish nearing the time of spawning than it was at earlier stages. In fully mature and spawning salmon the islets of Langerhans were relatively large and abundant (Fig. 26). The average size of the largest islets found in the sea fish was 89 μ in diameter while those of the spawning fish measured 554 μ.[10] Rarely was a large islet encountered in a sea salmon. Accompanying islet hypertrophy was a diminution in the amount of zymogenous tissue but the remaining cells appeared normal and contained many large deep staining granules. In most cases fat had largely disappeared from the pancreatic tissue by the time of full sexual maturity.

[10] Greene (1) stated that the islets were more prominent in spawning than in immature salmon but did not consider this to be hypertrophy.

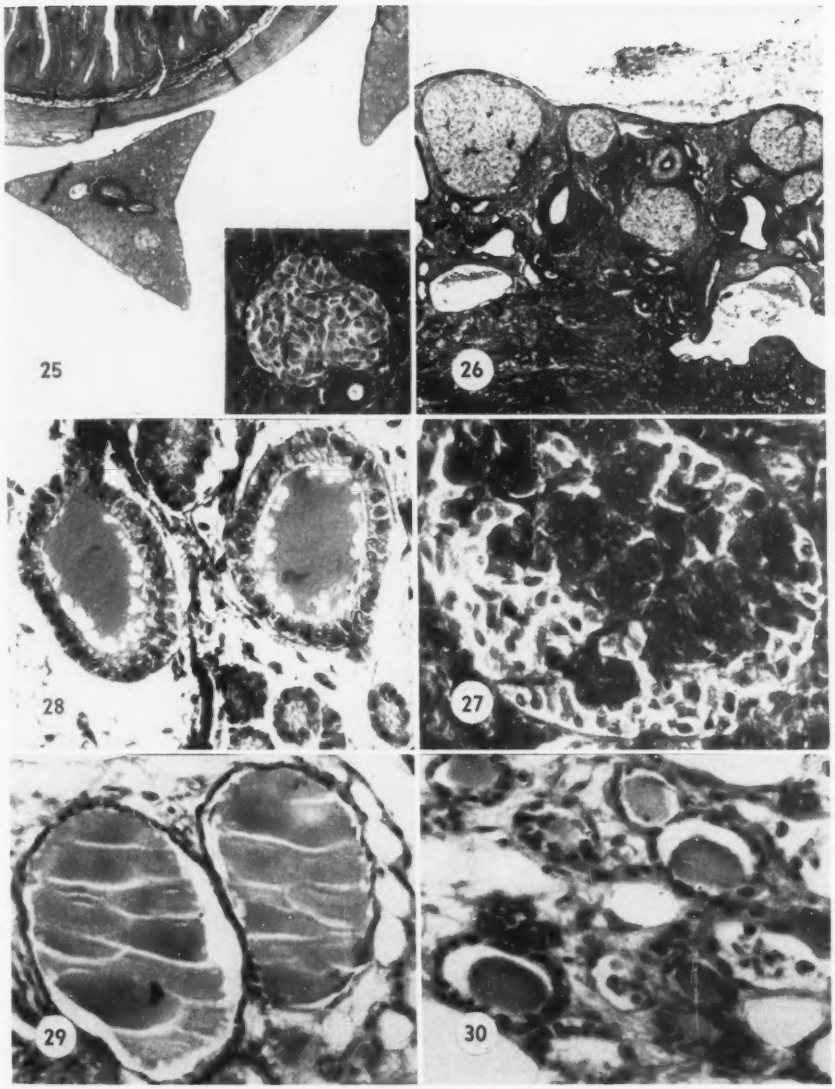

FIG. 25. Section of pancreas of immature sea salmon containing small islet of Langerhans. Paraldehyde, azan ×40. Enlarged inset (×175) shows cellular structure of islet.

FIG. 26. Pancreatic tissue of spawning king salmon—contains many hypertrophied islets. Gomori aldehyde fuchsin ×40.

FIG. 27. Islet of Langerhans stained with Gomori's aldehyde fuchsin and counterstained with Masson technique. Shows deep staining (heavily granulated) beta cells surrounded by alpha cells, largely degranulated. ×400.

FIG. 28. Thyroid tissue of immature sea king salmon showing increased follicular activity—cell height 13 μ. H. and E. ×175.

FIG. 29. Thyroid of spent king salmon. Follicle cells flattened; about 1 μ in thickness. H. and E. ×175.

FIG. 30. Different area of same thyroid as Fig. 29, showing degeneration of follicles. H. and E. ×400.

In the spawning salmon two types of islet cells were clearly distinguishable, deep-staining, well-granulated beta cells and the much lighter-staining mostly degranulated alpha cells (Fig. 27). The former outnumbered the latter. While these two cell types were less often detectable in the immature seafish, where the distinction could be made, the alpha cells seemed to be predominant. Alpha and beta cells have been described in a number of fishes (18) including rainbow trout (11).

Degenerative changes in the islets of Langerhans were uncommon in the spawning salmon but were observed in an occasional fish, and consisted of rupture of cell membrane and loss of granules of both alpha and beta cells.

Thyroid

Histologically the thyroid showed evidence of progressive decrease in activity during the spawning migration. Measurements of follicle cell height in ten king salmon taken in the sea and at the entrance to the river, gave an average of 6.5 μ. The greatest cell height occurred in a sea fish, namely 13 μ (Fig. 28). Ten migrating fish with gonads in various stages of maturation showed a follicle cell height of 4.4 μ while 13 spawning and spent salmon averaged 2.9 μ. The size of the follicles did not show much variation from sea to spawning ground nor did the amount of colloid in them. However in two salmon showing follicle cell heights above the average—one in the sea and one well up the river—the colloid was much diminished and vacuolated.

Atrophy and degeneration involving varying numbers of follicles were found in all thyroids of spawning fish. Evidence of degeneration was more clearly discernible in the less atrophic follicles. This ranged from loss of cytoplasm to disruption of the follicle wall and ultimate disappearance of the entire structure (Figs. 29 and 30).

Gonads

Testes. Examination of the testes of salmon at varying stages of maturation showed no evidence of any appreciable degenerative change up to the time of full sexual maturity, however, in the spawning fish marked degeneration was present. This has been described in the testes of sockeye salmon by Weisel (20). We have found similar changes in the king salmon and kokanee, i.e. vacuolization, loss of cytoplasm, caryolysis and complete disintegration of the cells lining the lobule walls which we believe comprise both spermatogonia and Leydig cell analogues (Figs. 31 and 32).[11]

[11] We have not tested for the presence of lipids in these cells of the salmon, but in a former study of rainbow trout, osmic acid fixation of the testis of spawning steelhead (anadromous rainbow trout) revealed an intense concentration of lipid material in the lobule-boundary cells which resembled the mammalian Leydig cell both in the morphology and lipid content (19). The process of gonad maturation in the Pacific salmon is similar to that occurring in rainbow trout.

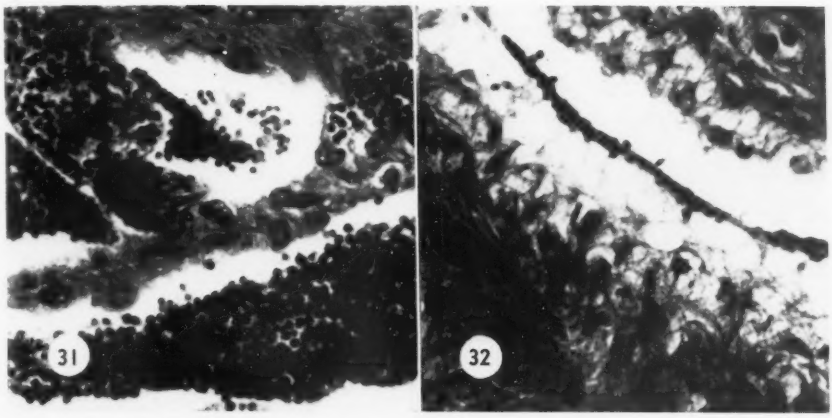

FIG. 31. Testis of salmon taken near spawning ground. Not quite sexually mature. Contents of lobule consists of both spermatids and spermatozoa. Cells lining wall of lobule are Leydig-cell homologues. Two cells show a small single eccentric nucleolus characteristic of these cells. No spermatogonia are present. H. and E. ×550.

FIG. 32. Testis of spent salmon. Empty lobules have collapsed. Walls greatly thickened. Cells lining lobule walls show marked degenerative change. H. and E. ×550.

Ovaries. As in the case of the testes, no degenerative changes were detected in the ovaries before spawning. After ovulation the cells of the granulosa, both still attached to the follicle wall and desquamated, showed various stages of degeneration from vacuolization to loss of nuclei, to complete dissolution. In several instances a few small, normal appearing, nucleated immature ova were present. Weisel (21) found many such oöcytes in the spent ovaries of sockeye salmon of Flathead Lake, as well as what appeared to be typical definitive germ cells. We did not find any cells similar to oögonia in king salmon.

Other Tissues

Muscle. The principal change in the body musculature of salmon is the massive storage of fat before beginning the spawning migration, which has been described by Greene (9). This fat is gradually used up and by the time of spawning scarcely any remains. Other than this no histological changes in the great lateral muscles have been detected. However, degeneration of individual fibers is present in the masseter muscle (9). This consists of pallor of the fiber, apparent fusing of the fibrils, vacuolization and finally complete disappearance of the fibrils. The cross striations are lost.

Cardio-vascular system. Widespread deteriorative changes of various kinds were found in the cardiovascular system, comprising degeneration of the heart muscle, intimal proliferation of the coronary arteries, thickening of the systemic artery walls with alterations in all three coats. A description of these changes will be given in a separate communication.

DISCUSSION

How is the foregoing array of histological changes exhibited by the spawning salmon to be interpreted? In view of the marked hyperplasia of the adrenal cortical tissue present in the sexually mature fish (3) and the accompanying greatly increased concentration of 17-hydroxycorticosteroids found by Hane and Robertson (22) to be present in the spawning salmon's blood, it would seem possible that the observed changes reflect the catabolic effects of these adrenal steroids. The change in concentration of 17-OHCS from the sea to the spawning grounds represented an increase of as much as 7-fold. The average in spawning females was 79 μg. %, in males 31 μg. %. If hyperadrenocorticism is the principal cause of the deterioration of the spawning salmon one might expect to find pathological changes similar to those exhibited by human patients suffering from Cushing's syndrome, the only other condition known in which continuous high concentrations of 17-OHCS persist over considerable periods of time. However, another possibility should be considered, namely, aging. Spawning signals the termination of the Pacific salmon's life cycle and the degenerative manifestations of the fish at this time, both external and internal, do suggest in certain respects the occurrence of a very rapid senescence. While there is no information available on histological changes in senile fish, the various tissues and organs of aging mammals, including man, have been studied extensively.

In order to bring out the similarities and differences in histological changes between spawning salmon and Cushing's syndrome plus experimental hyperadrenocorticism on the one hand and aging processes on the other, pertinent data are presented in Table 1. Space limitation has necessitated a very brief description of the type of change. Its general nature is indicated with particular reference to the kind of alteration occurring in the same organs of the salmon. Furthermore, in this condensed tabulation it has been impossible to designate degree of histologic change. Our findings would indicate that the changes occurring in the spawning salmon are, with the exception of those of the cardiovascular system, more pronounced and generalized than are the corresponding ones found in Cushing's syndrome, experimental hyperadrenocorticism or aging.

In comparing the changes found in Cushing's syndrome with those occurring in the spawning salmon, one finds striking resemblances as well as certain dissimilarities. Hyperplasia of the adrenal cortical tissue and degenerative changes in the pituitary are present in both—the latter are much more pronounced in the salmon than in the human being with Cushing's disease. Other internal organs which exhibit pathological alterations in patients with Cushing's syndrome are also found to be affected in the spawning salmon and for the most part with the same type of histologic change. Depletion of lymphocytes found in the spleen and thymus of the salmon has been otherwise described only in the latter organ in Cushing's

TABLE 1. HISTOLOGICAL CHANGES IN SPAWNING SALMON, CUSHING'S SYNDROME, EXPERIMENTAL HYPERADRENOCORTICISM AND AGING

	Spawning salmon	Cushing's syndrome	Experimental hyperadrenocorticism	Aging in mammals and man
Adrenal	hyperplasia and degeneration	hyperplasia (49) and tumors	hyperplasia	slight atrophy (23, 24)
Pituitary	degeneration (2)	hyaline change in basophils (45)	minimal degeneration (25, 26)	degeneration (23, 44)
Spleen	depletion of lymphocytes, fibrosis	no reported change	depletion of lymphocytes (27)	atrophy, depletion of lymphocytes, fibrosis (28, 29)
Thymus	involution, depletion of thymocytes	involution, (55) occasional tumor	depletion of thymocytes (30, 27)	involution (31)
Liver	degeneration	occasional fatty degeneration (53, 54)	no change (32)	slight degeneration (34, 33)
Kidney	degeneration	degeneration (47, 35)	degeneration (50, 46)	atrophy and degeneration (36, 37)
Pancreas	hypertrophy of islets	hypertrophy of islets variable (47)	hypertrophy of islets (6)	no distinctive change
Stomach	atrophy and degeneration	occasional ulcers (52)	atrophy of epithelium and occasional ulcers (43)	no change
Thyroid	atrophy and degeneration	atrophy of follicular epithelium (47)	atrophy of follicular epithelium (43)	involution (38)
Gonads	degeneration of testes	atrophy (39, 47)	degeneration (40)	atrophy and degeneration
Muscle	degeneration of masseter	atrophy (51)	no change	atrophy
Cardio-vascular system	degeneration and beginning arteriosclerosis	arteriosclerosis (49)	arteriosclerosis (41)	arteriosclerosis
Skin	hypertrophy	atrophy (39)	atrophy (42)	mostly atrophy; in some regions hypertrophy (34)

syndrome. However in experimentally produced hyperadrenocorticism, conspicuous lymphocyte depletion has been shown to take place in both spleen and thymus. Changes in the skin are quite different in spawning salmon and the Cushing's patient—hypertrophy in the former, atrophy in the latter. Thus, except for the skin the histologic picture presented by the spawning salmon resembles that occurring in mammals subjected to high concentrations of 17-hydroxycorticosteroids, either natural or experimental. Prolonged starvation which characterizes the salmon on its spawning migration may well accentuate the degenerative processes. An organ such as the liver, which appears to be quite resistant to the catabolic (or antianabolic) effects of adrenal corticosteroids in the experimental feeding animal, may react quite differently under conditions of starvation. That inanition of itself causes marked histological changes has been brought out by Baker (43) who showed that many of these alterations, principally atrophy and degeneration, are similar in nature to the changes induced by experimental hypercorticism.

Comparison of the histological changes in spawning salmon with those found in old animals reveals a general similarity except for the absence of adrenal hyperplasia and the lack of alteration in the stomach and pancreas. While in most old animals the liver shows very little change, senile rats

have been found to exhibit definite deteriorative processes (33). Modifications in the histology of some organs in aging mammals, e.g. the kidney, would seem to be chiefly secondary to degenerative changes in the blood vessels (36). However, atrophy of other tissues, skin, muscle and gonads appear to occur in the absence of any marked local vascular change.

The evidence which we have presented would suggest that the histological changes present in the spawning salmon bear a closer resemblance to those occurring in Cushing's syndrome and experimental hyperadrenocorticism than to those taking place in aging tissues. However, the fact that so many of the changes occurring in hyperadrenocorticism and aging are similar, raises the question as to the possibility of a common factor operating in both conditions. The possible relationship of adrenal cortical hormones to aging has been discussed at length by Solez (48) who pointed out the numerous aspects of premature aging exhibited by patients with Cushing's syndrome. However, in relation to the salmon we believe that it would be unwise to draw more than very tentative inferences concerning analogies between aging processes and degenerative changes in the spawning salmon until we have learned something about the characteristics of senile fish tissues.

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PRODUCTION OF HYPERTENSION IN ADRENALECTOMIZED RATS GIVEN HYPERTONIC SALT SOLUTION TO DRINK[1]

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ABSTRACT

The adrenal glands of the rat do not appear to be necessary for development of hypertension induced by administration of hypertonic NaCl solution as drinking fluid. Systolic blood pressures of these rats became elevated within 3 to 8 weeks after treatment began. The lowest concentration of salt solution which significantly elevated blood pressure was 1.75%. Renal hypertrophy accompanied the elevated blood pressure in most cases and cardiac hypertrophy in some cases. The life span of the adrenalectomized rats receiving hypertonic salt solution to drink was less than that of untreated adrenalectomized rats.

IT HAS been established without question that ingestion of excessive amounts of sodium chloride produces hypertension and renal lesions in rats whether the sodium chloride is ingested by way of food or fluid (1–3). It seems reasonable to implicate the adrenal cortex as a contributor to this type of hypertension in view of its well known role in the maintenance of electrolyte balance. However, Brandt *et al.* (4) observed that rats made hypertensive by administration of 2% sodium chloride solution for 6 weeks and then adrenalectomized, did not manifest a fall in blood pressure. Hence, it seems unlikely that the adrenal glands play a significant role in the *maintenance* of this type of hypertension.

The studies described here were performed to determine whether hypertension could be *induced* in adrenalectomized rats given hypertonic sodium chloride solutions to drink. When it was established that this was possible, a further objective was to determine the lowest concentration of sodium chloride solution which would produce hypertension in adrenalectomized rats.

METHODS

Four separate experiments were performed each of which utilized male rats of the Holtzman strain. All animals were kept in a thermoregulated room maintained at $25 \pm 1°$ C and illuminated from 8 A.M. to 6 P.M. The food used in all experiments was Purina laboratory chow. The drinking fluid was always a sodium chloride solution, the concentration of which varied from experiment to experiment and will be described below for each experiment. Blood pressures were measured by the microphonic manom-

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ter technique (5) but without anesthesia as described earlier (6). Other techniques pertinent to each experiment will be described below.

Experiment 1

Ten adrenalectomized rats weighing 330 to 350 gm. were used. Immediately after adrenalectomy each rat was placed in an individual cage and given choice between water and 0.9% NaCl solution as drinking fluids. Completely adrenalectomized rats are well known to manifest a spontaneous appetite for NaCl solution (7–9). When it was certain that the spontaneous salt appetite was present in each rat, measurement of food and fluid intakes began. These measurements, including body weight, were made daily throughout the 3 week control period and during the first 9 weeks of the experiment. From the 13th week on, intakes were measured every other week.

Systolic blood pressure was measured once during the control period and weekly thereafter. At the end of the control period 5 of the 10 rats, chosen at random, were given 2% NaCl solution as their sole drinking fluid (Group 1). The remaining 5 rats (Group 2) were given 0.9% NaCl solution as their sole drinking fluid. Ground Purina chow[2] was used as food for all rats during the experiment. The types of food and fluid containers used have been described in detail previously (10).

At the end of the 10th experimental week, the group of rats (Group 1) which was given 2% NaCl solution to drink was switched to 0.9% NaCl solution while Group 2, which was given 0.9% NaCl solution to drink, was switched to 2.0% NaCl solution. All measurements continued as before. At the end of the 19th experimental week, Group 2 was returned to 0.9% NaCl solution as drinking fluid. During weeks 26 to 28, all rats were given choice between 0.9% NaCl solution and distilled water as drinking fluids. The experiment ended on the 28th week.

Experiment 2

This experiment was performed to confirm and extend the results of Exp. 1 in which a 2.0% NaCl solution was observed to produce an elevation in the blood pressure of adrenalectomized rats. Twenty-five rats were used; all were adrenalectomized. They were divided into two groups. Group 1 consisted of 12 rats and Group 2 of 13. During a 2 week control period, all rats were given choice between 0.9% NaCl solution and water to drink to test completeness of adrenalectomy as in Exp. 1. Blood pressures and body weights were measured weekly during control period and throughout the 15 week experimental period. At the end of control period, Group 1 received 0.9% NaCl solution and Group 2 received 1.9% NaCl solution as their sole drinking fluid. The experiment was terminated after 15 weeks.

Experiment 3

The third experiment was carried out to extend the results of the second experiment in which 1.9% NaCl solution produced an elevation of blood pressure in adrenalectomized rats. This was deemed necessary in view of the fact that relatively few rats in Exp. 2 had survived the 15 week experimental period. In the third experiment three groups of rats were used. Group 1 contained 10 normal rats while Groups 2 and 3 were composed of 10 adrenalectomized rats each. During a two week control period, all rats were given choice between water and 0.9% NaCl solution to drink to assess completeness of adrenalectomy in the rats of Groups 2 and 3. Blood pressures were measured weekly during control period and throughout the 19 week experiment.

[2] Analysis of Purina chow by wet ashing with nitric acid and measurement of sodium and potassium concentration by flame photometry revealed a sodium content of 132.4 mEq./kg. food and a potassium content of 220.3 mEq./kg. food.

At the end of the control period, Groups 1 and 2 were given 0.9% NaCl solution and Group 3 was given 1.75% NaCl solution as sole drinking fluid. The experiment continued until the 11th week when 1.9% NaCl solution was substituted for the 1.75% NaCl solution given Group 3 previously. The rats were maintained on 1.9% NaCl solution for the remainder of experiment.

Beginning the 18th week all rats were anesthetized with pentobarbital sodium (30 mg./kg., intraperitoneally) and blood pressures measured as before to compare with blood pressures measured when the rats were unanesthetized. At the end of the 18th week all rats were subjected to a cold experiment. Completely adrenalectomized rats are unable to maintain their body temperatures as long as normal rats when restrained and exposed to air at 5° C (11). Consequently, they cool at a significantly faster rate. The rats were restrained as described in detail previously (11, 12) and a copper-constantan thermocouple was inserted 5 cms. into the colon. The thermocouple was led off to a potentiometer which recorded the temperature at one minute intervals. After a control period in air at 25° C, rats were placed in air at 5° C until their colonic temperatures fell to 26° C at which time they were freed, removed from the cold and rewarmed artificially. Colonic cooling rates (° C/hr.) were used to compare responses to cold among the groups since the relationship between colonic temperature and time is approximately linear within the range of colonic temperatures used here; i.e., 38 to 26° C (12, 13).

Experiment 3 was terminated at the end of the 19th week. All remaining rats were sacrificed at this time by ether inhalation and carefully examined for any possible remaining adrenal tissue. Heart, kidneys, testes, prostate, seminal vesicles, thyroid and thymus glands were removed, drained of blood or fluid, blotted and weighed on a torsion balance. Organ weights are expressed on a body weight basis; i.e., mg./100 gm. body weight.

Experiment 4

The fourth experiment was performed to determine more precisely the lowest concentration of NaCl solution which would produce an elevation in blood pressure over a 13 week experimental period. Thirty adrenalectomized rats were used. These rats were adrenalectomized one week prior to beginning the experiment and were given choice between 1.0% NaCl solution and distilled water as drinking fluids to assess completeness of adrenalectomy as in Exp. 1. All rats received ground Purina laboratory chow as food. Food and fluid intakes and body weight were measured daily throughout the experiment while blood pressure was measured weekly. At the end of the control period the rats were divided at random into 5 groups which received the following NaCl solutions as their sole drinking fluids: Group 1, 0.90%; Group 2, 1.25%; Group 3, 1.50%; Group 4, 1.75%, and Group 5, 2.00%. There were 5 rats per group in the first 4 groups and 10 rats in the 5th group. Measurements continued for 13 weeks at the end of which the experiment was terminated. All rats remaining at the end of experiment were sacrificed by ether inhalation and examined carefully for completeness of adrenalectomy. The same organs were removed as described in Exp. 3. These were weighed on a torsion balance and ratio of organ weight to body weight calculated for each organ. Simultaneous means of data taken from control and experimental groups were compared using the "t" test for the 95% confidence limit (14).

RESULTS

Experiment 1

Administration of 2% NaCl solution to adrenalectomized rats of Group 1 resulted in a rise in blood pressure first detectable 6 to 7 weeks later and

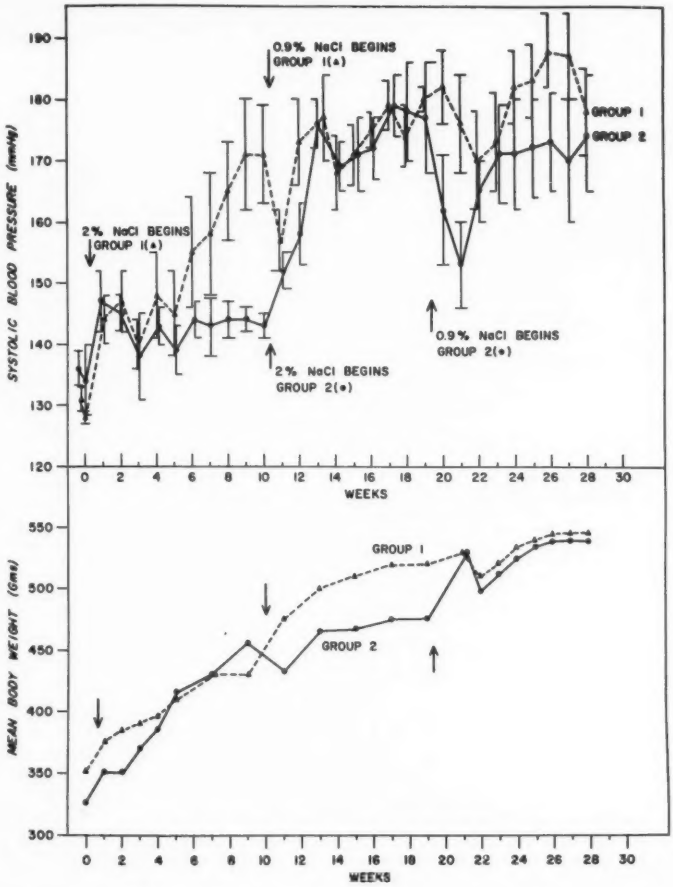

FIG. 1. The upper part of this figure illustrates mean systolic blood pressures throughout the experiment for the two groups of adrenalectomized rats used. ±1 standard error is set off at each mean. See text for explanation of this figure. The lower part of this figure illustrates mean body weight of the two groups of adrenalectomized rats used.

significantly higher than that of controls during the 8th week (Fig. 1). Blood pressure of rats receiving 0.9% NaCl solution was not affected. When salt solutions were switched at the end of the 10th week, mean systolic blood pressure of rats which had received 2% NaCl solution (Group 1) fell transiently but returned to previous high levels the following week. The mean systolic blood pressure of rats in Group 2, which was switched to 2% NaCl solution at the end of the 10th week, began to rise. The first detectable rise occurred within 1 week after administration of 2% NaCl solution. Mean systolic blood pressure level of this group rose to levels of 170 to 175 mm. Hg within 3 weeks. At the end of the 19th week, Group 2 was given

0.9% NaCl solution in place of 2% NaCl solution. It was observed again that blood pressure level fell during the next two weeks but returned to previous high levels during the following two weeks. Blood pressure remained elevated thereafter. Thus, 2% NaCl solution produced an elevation of blood pressure in both groups of rats. The elevated pressure failed to return to normal levels even though the concentration of the salt solution was returned to isotonicity.

The lower graph of Figure 1 illustrates that growth of the rats receiving 2% NaCl solution was retarded. When this hypertonic solution was switched to the isotonic solution, growth rate accelerated. The sodium ingested (mEq./100 gm. BW/day) during each period of the experiment is shown in Table 1. Data from Group 1 are shown on the left and those from Group 2 are shown on the right of table. Total fluid intake and virtual sodium concentration are also shown for each group. The latter is calculated as (total sodium intake/total fluid intake $\times 1000$) and is expressed as mEq./liter.

During the 3 week control period (weeks -3 to -1 inclusive), all rats showed a spontaneous NaCl appetite and were considered completely

TABLE 1. THE EFFECT OF HYPERTONIC NaCl SOLUTION ON FOOD AND FLUID INTAKES OF ADRENALECTOMIZED RATS

	Group 1					Group 2				
	Na intake (mEq./100 gm. BW/day)			Total fluid intake (ml./100 gm.	Virtual Na intake conc.	Na intake (mEq./100 gm. BW/day)			Total fluid intake (ml./100 gm.	Virtual Na intake conc.
Week	Food	Fluid	Total	BW/day)	mEq./l.	Food	Fluid	Total	BW/day)	mEq./l.
Choice of 0.9% NaCl soln. and water						*Choice of 0.9% NaCl soln. and water*				
−3	0.81	2.48	3.29	18.1	182	0.78	3.44	4.22	23.7	178
−2	0.79	2.43	3.22	17.5	184	0.82	3.42	4.24	23.5	180
−1	0.74	2.42	3.16	17.3	183	0.80	3.27	4.07	22.6	180
2% NaCl soln. given (no choice) Week 1						*0.9% NaCl soln. given (no choice)*				
1	0.67	5.85	6.52	16.9	386	0.72	2.79	3.51	18.6	189
2	0.63	6.04	6.67	17.5	381	0.77	2.78	3.55	18.5	192
3	0.60	5.94	6.54	17.2	380	0.65	2.61	3.26	17.4	187
5	0.61	6.66	7.27	19.3	377	0.63	2.40	3.03	16.0	189
7	0.60	6.14	6.74	17.8	379	0.63	2.30	2.93	15.3	192
9	0.58	6.32	6.90	18.3	377	0.69	1.95	2.64	13.0	203
0.9% NaCl soln. given (no choice) Week 10						*2% NaCl soln. given (no choice)*				
13	0.74	1.97	2.71	13.1	207	0.37	5.52	5.89	16.0	368
15	0.58	1.67	2.25	11.1	203	0.55	5.07	5.62	14.7	382
17	0.60	1.98	2.58	12.8	202	0.51	6.11	6.62	17.7	374
19	0.56	1.86	2.42	12.0	202	0.55	6.39	6.94	18.5	375
						0.9% NaCl soln. given (no choice)				
21	0.61	2.22	2.83	14.3	198	1.05	2.76	3.29	17.8	185
23	0.60	1.97	2.57	12.7	202	1.33	1.78	2.45	11.5	213
Choice of 0.9% NaCl soln. and water Week 26						*Choice of 0.9% NaCl soln. and water*				
26	0.63	1.10	1.73	10.1	171	1.28	0.84	1.48	8.7	170
27	0.61	1.17	1.78	10.8	165	1.12	0.92	1.48	8.4	176

drenalectomized. The total sodium intake was relatively consistent within ach group but approximately 1 mEq./100 gm. BW/day higher in Group 2 han in Group 1. The virtual intake concentrations of the two groups, how- ver, are nearly identical. When 2% NaCl solution was given to Group 1 weeks 1 through 9), total sodium intake more than doubled while total luid intake remained near control levels. Similar results were observed hen Group 2 was given 2% NaCl solution to drink (weeks 10 through 19). Thus, doubling total sodium intake and virtual sodium intake concentra- ion above levels observed during the control period was accompanied by n elevation in blood pressure.

Experiment 2

A slightly lower concentration of NaCl solution (1.9%) than was used in Exp. 1 increased blood pressure significantly within 4 to 5 weeks after initi- ation of treatment. Blood pressure continued to rise until about the 11th week of experiment. Thereafter, blood pressure level remained relatively constant (Fig. 2A). Blood pressures of rats receiving 0.9% NaCl solution to drink were not affected.

Body weights of the two groups of rats were similar throughout the experiment (Fig. 2B).

Forty-six per cent (6 of 13) of the rats receiving 1.9% NaCl solution as their sole drinking fluid survived 10 weeks of this regimen. By the end of the experiment (15 weeks) 23% remained (Fig. 2C) while 50% of the rats receiving 0.9% NaCl solution to drink survived the experiment. Though too few rats survived to test for completeness of adrenalectomy, all rats used had a NaCl appetite at the beginning of the experiment.

Experiment 3

The blood pressure of adrenalectomized rats receiving 1.75% NaCl solution in their diet showed an observable rise above the level of adrenalectomized rats within 6 to 7 weeks after initiation of the salt treatment (Fig. 3A). Blood pressure reached a level of approximately 155 mm. Hg and tended to remain at this level. When 1.9% was substituted for 1.75% NaCl solution (week 11), blood pressures rose to approximately 162 mm. Hg within 2 weeks. Blood pressures remained at this level. The difference between these two blood pressure levels was not statistically significant. Adrenalectomized rats receiving 0.9% NaCl solution to drink maintained a blood pressure level of 140 to 145 mm. Hg throughout the experiment. Blood pressure level of control non-adrenalectomized rats varied from 145 to 150 mm. Hg.

Control, non-adrenalectomized rats grew at a faster rate than either of the other two groups (Fig. 3B). Adrenalectomized rats given 1.75% NaCl solution to drink grew at the slowest rate. Adrenalectomized rats receiving 0.9% NaCl solution to drink grew at an intermediate rate.

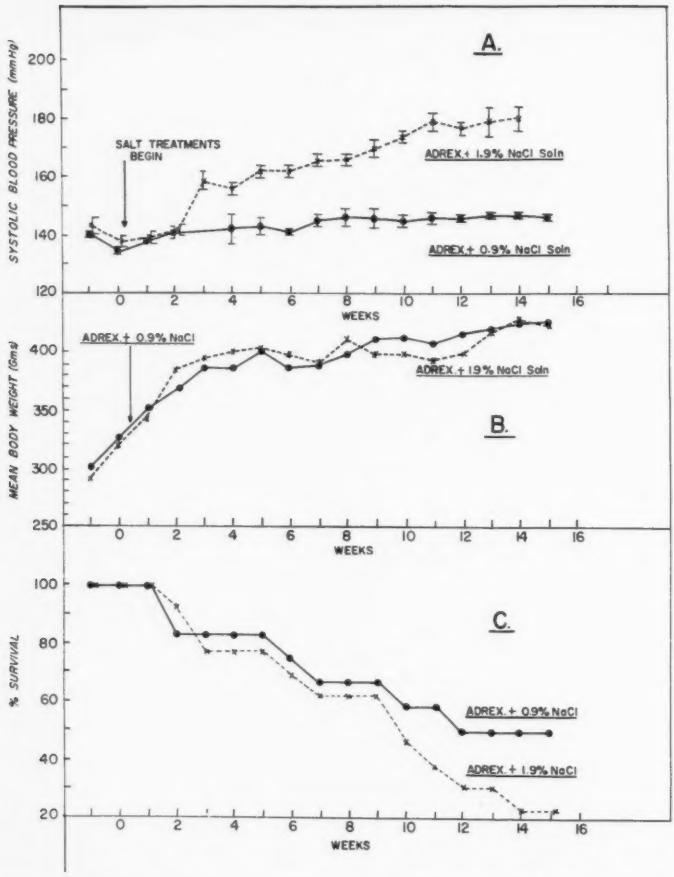

FIG. 2. A. Mean systolic blood pressures of adrenalectomized rats (X) given 1.9% NaCl solution as their sole drinking fluid, and adrenalectomized rats (●) given 0.9% NaCl solution as their sole drinking fluid are shown throughout the experiment. ±1 standard error is set off at each mean.

B. Mean Body weight of the two groups of rats throughout the experiment.

C. Per cent survival of the two groups of rats throughout the experiment.

Fifty per cent (5 of 10) of the adrenalectomized rats receiving the hypertonic salt solution to drink were alive at the end of experiment (Fig. 3C). This increased survival rate above that of Exp. 3 is attributed to initiating the experiment with 1.75% NaCl solution and then increasing the salt concentration to 1.9%. By far a greater mortality (20% of the original group) occurred after initiation of 1.9% NaCl solution as the sole drinking fluid than occurred when 1.75% NaCl solution was given. Seventy per cent (7 of 10) of the adrenalectomized rats receiving 0.9% NaCl solution to

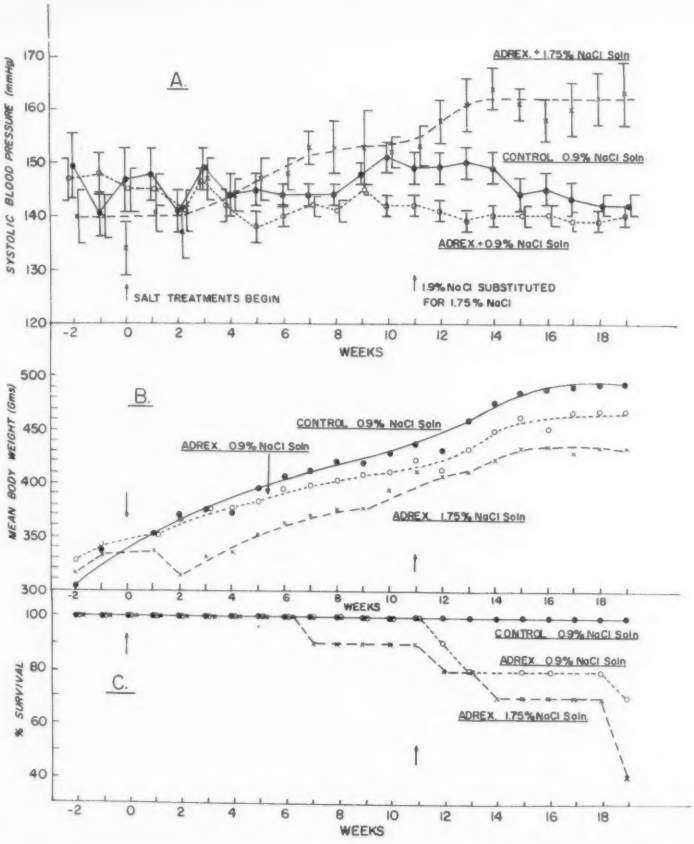

FIG. 3. A. Mean systolic blood pressures of adrenalectomized rats receiving 1.75% NaCl (X) and 0.9% NaCl solution (O) as their sole drinking fluid are shown throughout the experiment. Blood pressures of a non-adrenalectomized group receiving 0.9% NaCl solution (●) as its sole drinking fluid are also shown throughout the experiment. ±1 standard error is set off at each mean.

B. Mean body weights of the three groups of rats throughout the experiment.

C. Per cent survival of the three groups of rats throughout the experiment.

drink survived the experiment while all control, non-adrenalectomized rats survived the same period.

During the last week of the experiment, all rats were subjected to a cooling test, the results of which are shown in Table 2. Both groups of adrenalectomized rats had significantly lower colonic temperatures prior to exposure to cold. Both groups of adrenalectomized rats cooled significantly faster than control, non-adrenalectomized rats. These results, taken together, strongly suggest that the rats were completely adrenalectomized.

TABLE 2. COLONIC COOLING RATES AND INITIAL COLONIC TEMPERATURES OF RATS USED IN EXPERIMENT 3

Experimental group	No. of rats	Mean body wt. (gm.)	Initial colonic temp. (° C)	P.[2]	Colonic cooling rate (° C/hr.)	P.	% increase in CCR above controls
Control + 0.9% NaCl Soln.	10	490	38.3 ±0.1[1]		1.56 ±0.20		—
				.05		< .01	
Adrex + 0.9% NaCl Soln.	8	453	37.9 ±0.2		3.82 ±0.32		145%
				< .01		< .01	
Adrex + 1.75% NaCl Soln.	7	416	37.7 ±0.1		3.63 ±0.42		133%

[1] ± standard error of mean.
[2] Probability value.

Three of 7 adrenalectomized rats receiving hypertonic salt solution to drink died within 2 days after the cooling test. One adrenalectomized rat receiving isotonic salt solution to drink also died within 1 day after cooling.

At the beginning of the 18th week, blood pressures were measured on all rats while anesthetized. The results appear in Figure 3A as the blood pressures for week 18 of the experiment. It may be observed that the blood pressure levels measured while the rats were anesthetized compare favorably with those taken immediately before and after when the rats were not anesthetized. Hence, the elevated pressure of rats receiving hypertonic salt solution to drink does not appear to be maintained by neurogenic stimuli.

All adrenalectomized rats which died during the experiment as well as those sacrificed at the end of the experiment were autopsied and examined carefully for possible adrenal tissue. In none of these rats was any adrenal tissue observed grossly. The organs removed at autopsy were weighed and the ratios of organ weight to body weight appear in Table 3. Either isotonic or hypertonic NaCl solution increased significantly ratio of heart weight to body weight above that of control, non-adrenalectomized rats. Ratios of kidney weight to body weight and testis weight to body weight were sig-

TABLE 3. ORGAN WEIGHT TO BODY WEIGHT RATIO OF CERTAIN ORGANS OF THE RATS IN EXPERIMENT 3

Experimental group	No. of rats	Mean body wt. (gm.)	Organ weight to body weight ratio (mg./100 gm. BW): Heart	Kidneys	Testes	S. vesicles	Prostate	Thyroid	Thymus	Adrenal
control +0.9% NaCl Soln.	10	480	298.4 ±14.7[1]	811.3 ±23.1	758.0 ±23.2	138.4 ±10.5	139.6 ±7.9	4.5 ±0.8	54.2 ±3.9	11.6 ±1.2
Adrex +0.9% NaCl Soln.	10	387	363.5* ±13.6	906.0 ±40.7	865.5 ±47.4	129.9 ±7.3	112.3 ±8.5	8.6** ±0.8	63.1 ±7.2	—
Adrex +1.75% NaCl Soln.	6	336	371.8* ±14.9	1035.7** ±61.9	979.2* ±74.6	158.6 ±11.2	137.7 ±8.2	8.5** ±1.0	62.3 ±8.7	—

[1] ±1 standard error of mean
* Significantly different from controls ($P < .05$).
** Difference from controls highly significant ($P < .01$).

ificantly increased above levels of control rats by treatment of adrenal-ctomized rats with 1.75% NaCl solution. Ratio of thyroid weight to body veight was increased significantly by salt treatment in both groups of drenalectomized rats. Other organs were not influenced by the treatment.

Thus, NaCl solutions ranging from 1.75 to 1.9% are able to elevate blood pressure of adrenalectomized rats significantly above that of control rats receiving 0.9% NaCl solution. An increased heart size and thyroid size were observed in both groups of adrenalectomized rats. The group receiving the hypertonic NaCl solution had an increased kidney size as well as an increased testicular size.

Experiment 4

Blood pressures of adrenalectomized rats receiving 0.90, 1.25 or 1.50% NaCl solutions as their sole drinking fluids tended to rise somewhat with time although the rise was slight in all cases except that of the rats receiving 1.50% NaCl solution (Fig. 4A). During the last 4 weeks of experiment, blood pressure of this group reached levels of about 150 mm. Hg. However, variability of blood pressures within this group was large. In part B of Fig. 4 are shown the blood pressures of adrenalectomized rats receiving 1.75 or 2.00% NaCl solutions to drink. Blood pressures of the rats receiving 0.90% NaCl solution to drink are shown for reference. Both groups receiving hypertonic salt solution to drink showed elevations of blood pressure observable 3 to 4 weeks after initiation of treatment. Blood pressures of both groups continued to rise in a similar fashion and reached levels of 165 to 170 mm. Hg by end of experiment.

Table 4 illustrates the mean daily total sodium intake, total fluid intake and virtual sodium intake concentration of each group of rats for the 13 week experimental period. The total sodium intake increased with increasing concentration of NaCl solution. The sodium ingested by way of food remained relatively constant from group to group. Total fluid intake was similar from group to group excepting those rats receiving 2.00% NaCl solution. Their virtual sodium intake concentration was similar to that of the rats receiving 1.75% NaCl solution. These two groups also showed similar rises in blood pressure.

Administration of 2.00% NaCl solution to adrenalectomized rats in this experiment was accompanied by a large mortality. Six of 10 rats were dead by the 11th week of the experiment. Two of 5 rats receiving 1.50% NaCl solution died during the 5th week of the experiment.

The rats which died during the experiment as well as those sacrificed at the end were autopsied and examined carefully for possible adrenal tissue. None was found in these rats. Of the organs removed and weighed only the kidneys of rats receiving 1.50 and 2.00% NaCl solution to drink were significantly greater than those of control adrenalectomized rats receiving

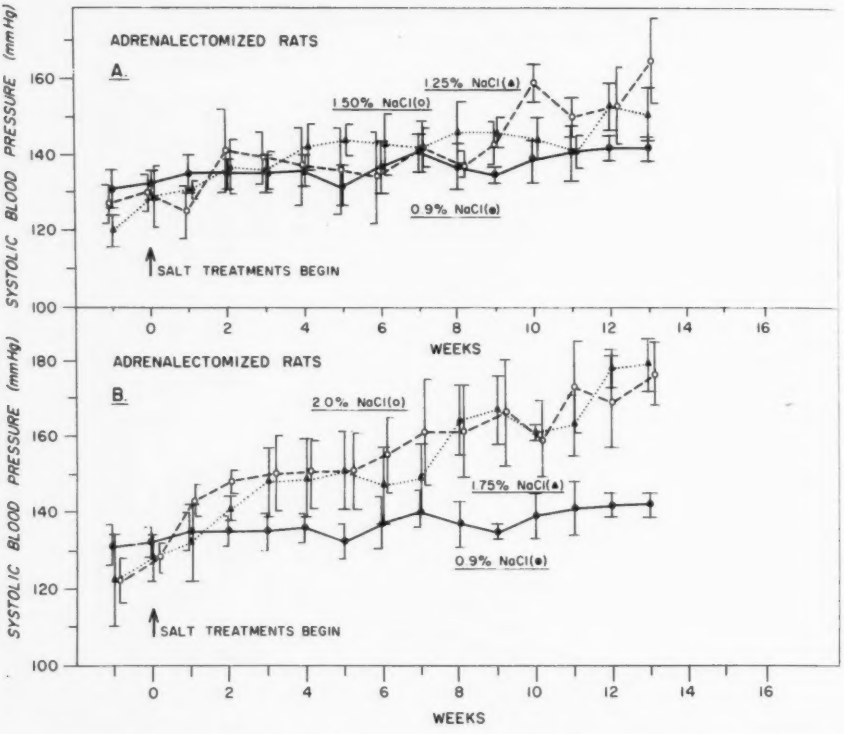

FIG. 4. A. Mean systolic blood pressures of adrenalectomized rats receiving 0.90% NaCl solution (●); 1.25% NaCl solution (▲) and 1.50% NaCl solution (O) as their sole drinking fluid are shown throughout the experiment. ±1 standard error is set off at each mean.

B. Mean systolic blood pressure of adrenalectomized rats receiving 0.90% NaCl solution (●); 1.75% NaCl solution (▲) and 2.00% NaCl solution (O) as their sole drinking fluids are shown throughout the experiment. ±1 standard error is set off at each mean.

0.9% NaCl solution (Table 5). It may be noted that thyroid size tended to increase in the hypertonic salt-treated groups. There was considerable variability of thyroid size within each group and the differences from the level of control adrenalectomized rats were not significant. However, the results appear to bear out those observed in Exp. 3 with respect to the thyroid gland. In Exp. 3 comparison was made between thyroid size of adrenalectomized rats receiving either 0.9 or 1.9% NaCl solution with that of non-adrenalectomized rats receiving 0.9% NaCl solution to drink. *Both* groups of adrenalectomized rats showed significant increases in thyroid size. The adrenalectomized rats of Exp. 4 all had thyroid glands which were significantly larger than those of non-adrenalectomized, control rats of

TABLE 4. MEAN SODIUM INTAKES OF 5 GROUPS OF ADRENALECTOMIZED RATS GIVEN PURINA CHOW AS FOOD AND DIFFERENT CONCENTRATIONS OF NaCl SOLUTION TO DRINK

Experimental group	Mean Na intake (mEq./100 gm. B.W./day)[1]			Total fluid intake ml./100 gm. BW/day)	Virtual Na intake conc. (mEq./l.)	Body weight (gm.)	
	Food	Fluid	Total			Initial	Final
0.90% NaCl (171 mEq./l.)	0.71	2.26	2.97	13.2	225	334	424
1.25% NaCl (214 mEq./l.)	0.67	3.05	3.72	14.2	262	343	442
1.50% NaCl (256 mEq./l.)	0.72	4.03	4.75	15.7	303	337	475[2]
1.75% NaCl (300 mEq./l.)	0.67	4.26	4.93	14.2	347	318	402
2.00% NaCl (342 mEq./l.)	0.63	10.51	11.14	30.8	362	302	352[3]

[1] Mean for the 13 week experimental period.
[2] Two rats died during the 5th experimental week.
[3] Six of 10 rats were dead by the 11th experimental week.

Exp. 3 but similar to those of the adrenalectomized rats of Exp. 3. It is interesting that the presence of adrenal glands should inhibit the increase in thyroid size attendent upon treatment with NaCl solutions.

DISCUSSION

Administration of hypertonic NaCl solution as the sole drinking fluid to adrenalectomized rats is accompanied by a rise in systolic blood pressure which occurs within 3 to 8 weeks after treatment begins. The lowest concentration of salt solution which significantly elevates blood pressure appears to be 1.75% (Fig. 3). It is possible that administration of lower concentrations for longer periods of time will also result in blood pressure ele-

TABLE 5. ORGAN WEIGHT TO BODY WEIGHT RATIO OF CERTAIN ORGANS OF THE RATS IN EXPERIMENT 4

Experimental group	No. rats	Mean body wt. (gm.)	Organ weight to body weight ratio (mg./100 gm. BW):						
			Heart	Kidneys	Testes	S. vesicles	Prostate	Thyroid	Thymus
0.90% NaCl	5	424	309.0 ±15.4[1]	765.8 ±17.5	821.6 ±85.0	142.5 ±14.0	128.1 ±13.8	8.14 ±0.88	67.4 ±8.2
1.25% NaCl	5	442	315.7 ±9.7	760.8 ±39.4	747.0 ±16.7	181.1 ±11.7	155.0 ±5.3	9.31 ±1.19	77.2 ±8.8
1.50% NaCl	5	475	308.8 ±19.0	862.7* ±34.4	799.2 ±75.0	145.6 ±16.3	165.5 ±31.5	11.36 ±1.20	67.0 ±9.9
1.75% NaCl	5	402	300.5 ±7.9	855.4 ±38.7	980.1 ±47.9	142.0 ±10.2	132.3 ±21.8	10.36 ±0.77	52.1 ±2.3
2.00% NaCl	6	352	372.2 ±37.2	991.6¶ ±62.5	957.0 ±95.4	151.2 ±20.1	152.0 ±21.1	11.50 ±2.11	62.8 ±11.0

[1] ±1 Standard error of mean.
* Difference from control (0.90% NaCl group) significant ($P < .05$).
¶ Difference from control highly significant ($P < .01$).

vation. Indeed, Heroux and Dugal (15) and Koletsky (16) reported elevation of blood pressure and occurrence of renal lesions in "normal" rats given isotonic saline as drinking fluid for extended periods of time. It seems likely that the extent of pathological effects produced is related both to the concentration of the NaCl solution given and to the duration of treatment.

The adrenalectomized rats given hypertonic NaCl solution to drink showed renal hypertrophy in most (Tables 3 and 4) and cardiac hypertrophy (Table 3) in some cases. The adrenalectomized rats of Exp. 3 which were given 0.9% NaCl solution to drink also developed cardiac hypertrophy to approximately the same level as that of the adrenalectomized rats given hypertonic NaCl solution to drink. This increased heart weight is surprising in view of the fact that the systolic blood pressure of this group was never elevated above control levels. It is difficult to state whether this condition might have accompanied excessive retention of sodium with consequent retention of water, increase of vascular volume and cellular edema. Unfortunately, water contents of the organs removed at autopsy were not determined.

One might suspect that the adrenal glands play a role in the development and maintenance of salt hypertension in view of their known activity in maintenance of electrolyte balance. However, treatment of normal rats with saline has been reported by several groups of investigators to induce atrophy and lipid depletion of the zona glomerulosa of the adrenal cortex (17–19). The zona glomerulosa is presumed to be the site of production of mineralocorticoids which have known pressor properties. Thus, even in animals with intact adrenals it would appear unlikely that overproduction of mineralocorticoids plays a major role in salt hypertension. The results of these experiments in which hypertension was shown to develop in adrenalectomized rats, bear out this thesis. Further, the studies of Brandt *et al.* (4) have shown that salt hypertension persists in rats that are adrenalectomized after hypertension has developed. The mechanism resulting in eventual hypertension under these conditions is poorly understood. Sensitization by salt to circulating pressor agents may be a contributing factor (20, 21). Accumulation of salt within the walls of arterioles with subsequent reduction of lumen size may represent still another factor (22). The latter possibility is an especially attractive one in view of the studies of Roemmelt *et al.* (23) who observed that adrenalectomized dogs given hypertonic NaCl solutions intravenously retained more sodium than normal controls rather than excreting it in excess as one might expect. Adrenalectomized rats given an isotonic saline load intravenously also require longer periods of time for excretion of the load than normal controls (24). It is possible that continued salt administration leads to positive salt balance in these animals with consequent increase in vascular volume, cellular edema and increased peripheral resistance.

An increased thyroid size accompanying salt administration was ob-

erved even in rats given isotonic saline as their sole source of drinking luid (Table 3). This observation is of interest in view of the studies of Hibbard (25) and Axelrad *et al.* (26). These investigators observed that ingestion of large amounts of NaCl increased thyroid activity as assessed by yperplasia, by histological changes characteristic of hypersecretion and y increased radioactive iodine uptake. Isler *et al.* (27) also observed thyoid stimulation in mice given 1% NaCl solution to drink. In addition, reatment of normal rats receiving 1.75% NaCl solution to drink with the nti-thyroid drug, propylthiouracil, prevented the expected elevation of lood pressure (28). Hence, it is possible that the thyroid gland may paricipate in the development of this type of hypertension.

In addition to the possibilities suggested above, salt hypertension may ccompany continued maximal concentrating effort of the kidneys. The act that hypertension developed only at concentration levels exceeding .75% tends to support this suggestion.

In such a study as this it is important to be assured that the rats used were completely adrenalectomized. When normal, non-adrenalectomized rats were used as controls, it was observed that growth rate of adrenalectomized rats was less than that of the normal rats (Fig. 3). This is characteristic of completely adrenalectomized rats, even those given NaCl solution to drink (29, 30). In addition, all rats used in these experiments showed a spontaneous NaCl appetite in which 90% or more of their total fluid intake was ingested as isotonic NaCl solution. Sufficient rats remained at the end of the third experiment to measure initial colonic temperatures and rates of cooling when exposed to cold air. The results of these experiments indicated that the rats were completely adrenalectomized in that the adrenalectomized rats had a lower colonic temperature prior to cold exposure and a faster cooling rate. These findings are characteristic of adrenalectomized rats (11). In addition, all rats that died or were sacrificed were carefully examined for possible accessory adrenal tissue. It is felt that the rats used were completely adrenalectomized.

It is uncertain whether a counterpart of this type of hypertension exists in humans. However, it has been observed that ingestion of 25 to 60 gm./day of table salt for 23 days produced a significant elevation of systolic and diastolic blood pressure in one human subject (31). Other evidence exists which suggests that individuals living in the Bahama Islands, an area in which drinking water contains a high concentration of sodium (.06M./l.), have higher blood pressure levels at a given age than do citizens of the United States (32). These results stress the importance for an understanding of the role sodium chloride may play in the etiology of hypertension.

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AMPHIBIAN OVULATION *IN VITRO* INDUCED BY MAMMALIAN PITUITARY HORMONES AND PROGESTERONE[1]

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ABSTRACT

The effect of various hormones on ovulation *in vitro* was studied with ovarian fragments from *Rana pipiens*. It was found that progesterone, interstitial cell-stimulating hormone and growth hormone induced ovulation under the conditions of the experiments, whereas follicle-stimulating hormone and prolactin were inactive.

ALTHOUGH numerous studies have been carried out to establish whether mammalian hormones are effective in inducing ovulation in amphibians *in vivo*, comparatively few investigations have appeared dealing with this problem *in vitro*. *In vitro* ovulation in amphibians was first observed by Rugh (1), who used the excised ovaries of a pituitary-injected *Rana pipiens*. The effect of pituitary extracts of the same species on *in vitro* ovulation in amphibians was also studied by Heilbrunn *et al.* (2) and others (3, 4, 5, 6, 7, 8). That steroid hormones may play an important role in the process of amphibian ovulation was suggested by the results of various *in vitro* experiments carried out with the South African clawed toad *Xenopus laevis* (9, 10, 11).

In the excised ovaries of *Rana pipiens*, both untreated and injected with extracts of amphibian pituitaries, cortisone has been shown to facilitate ovulation (12). To our knowledge the effect of mammalian pituitary gonadotropins on amphibian ovaries has so far been investigated only by Wright and Hisaw (13). These authors tested the ovulation-inducing potency of crude and fractionated anterior pituitary extracts from sheep, on the excised ovaries of *Rana pipiens*. Since considerable progress has recently been made in the purification of adenohypophyseal hormones, it was deemed worthwhile to reinvestigate in *Rana pipiens* the ovulation-inducing potency of highly purified preparations of mammalian anterior pituitary hormones. In addition, the effect of progesterone in this species was investigated.

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[2] Rockefeller Foundation Fellow on leave from the University of Utrecht, Netherlands.

MATERIAL AND METHODS

The experiments were carried out during the period from March 15 to April 15[3] with organs and tissues from female *Rana pipiens* weighing 40–70 gm. Every week a batch of frogs was brought in by a dealer, who received them from frog catchers in the Upper Mississippi Valley. The animals were kept in a quiet semi-dark place at room temperature.

A standard frog pituitary extract was obtained in the following manner: female frogs were decapitated, the pituitaries were removed, and the anterior lobes were separated from the intermediate lobes and freed from the endolymphatic tissues. The anterior lobes were ground and mixed with Ringer's solution so that 1 ml. of this suspension contained the equivalent of 1/4 of an anterior lobe. The suspension was kept frozen. To sensitize the ovaries, 1 ml. of the standard extract was injected into the dorsal lymph sac of the frogs. Eighteen hours later these frogs were decapitated, and their ovaries were excised and divided into 6–12 nearly equal parts, depending on the size of the ovaries. Each part was washed and thoroughly shaken in Holtfreter's solution before being immersed in 10 ml. of test fluid. Twenty-two to 24 hours after immersion all the ovarian fragments were shaken and the free eggs were counted.

To determine the ovulation-inducing potency of a substance, the number of eggs which had been released from an ovarian fragment immersed in 10 ml. of Holtfreter's solution containing that substance was compared with the number of eggs which had ovulated from another fragment of the same ovaries that had been immersed in a Holtfreter's solution containing 50% of the animals' own mashed anterior pituitary lobe. In each experimental series, the ovaries of 3 or 4 frogs were tested at the same time. The top, bottom and center portions of these ovaries were placed alternatingly in the various solutions. The number of eggs which had ovulated from the ovarian fragment immersed in the suspension containing 50% of the frogs' own anterior pituitary lobe was defined as 100% ovulation, in each instance. A suspension of the animals' own brain mashed in 10 ml. of Holtfreter's solution, or Holtfreter's solution alone, served as controls.

Interstitial cell-stimulating (ICSH), follicle-stimulating (FSH), lactogenic (prolactin, LTH) and growth (GH) hormones were prepared from sheep pituitary glands by procedures previously described (14, 15, 16, 17). Crystalline progesterone was generously supplied by Parke, Davis and Company.

The progesterone solutions were prepared by shaking progesterone crystals in Holtfreter's solution for 24 hours at room temperature. The solution was then centrifuged and the supernatant fluid served as test solution. The progesterone concentration of this fluid was determined[4] by extracting the steroid with redistilled dichloromethane and computing from the absorption spectrum of the hormone in methanol, read in the Beckman model D.U. spectrophotometer.

RESULTS

When fragments of excised ovaries were immersed in various concentrations of progesterone in Holtfreter's solution, it was found that this steroid causes ovulation, the minimal effective concentration being approximately 0.6 μg./ml. (Table 1). In addition, it was demonstrated that progesterone in a concentration of approximately 6 μg./ml. also induced ovulation in the ovaries of animals which had not been pretreated with the standard extract of amphibian pituitaries.

[3] This is the period just before the mating season of this frog.

[4] The authors wish to thank Dr. Hans Carstensen for his invaluable advice in making this determination.

TABLE 1. OVULATION RESPONSE OF OVARIAN FRAGMENTS OF PRETREATED *Rana pipiens* AS INDUCED BY PROGESTERONE

Agent in Holtfreter's solution (10 ml.)	Steroid concentration	Number of experiments	Number of positive responses	Average per cent of eggs ovulated
	μg./ml.			
Progesterone	6	8	8	56
Progesterone	0.6	8	5	13
Progesterone	0.06	8	0	0
Frog's own pituitary lobes	0	8	8	100
Frogs' own brain	0	8	0	0
Holtfreter's solution only	0	8	0	0

Table 2, which summarizes the results of experiments in which pituitary hormones were used, shows that ICSH and GH are quite potent in inducing ovulation *in vitro*, whereas FSH and LTH are inactive in this respect. It was thought at first that a certain ratio of ICSH and GH would be required to induce the maximal degree of ovulation; however, the results of experiments carried out with combinations of various quantities of these two hormones did not support this hypothesis.

To investigate whether the presence of FSH may facilitate the ovulation induced by ICSH or GH alone, combinations of these hormones in various concentrations were tested. The results of these experiments showed no marked synergistic effect on the part of the FSH. A seasonal increase in the sensitivity of the ovaries to progesterone, ICSH and GH during these experimental series was noticed; however, injections of stand-

TABLE 2. OVULATION RESPONSE OF OVARIAN FRAGMENTS OF OVARIES FROM PRETREATED *Rana pipiens* AS INDUCED BY VARIOUS SHEEP PITUITARY HORMONES

Preparation	Concentration of the hormone	Number of experiments	Number of positive responses	Per cent of positive responses	Average per cent of free eggs
	μg./ml.				
ICSH	200	2	2	100	73
	100	20	17	85	54
	50	11	10	91	44
	20	10	9	90	12
	10	7	3	43	5
	5	7	2	30	2
FSH	100	12	0	0	0
	50	3	0	0	0
	20	4	0	0	0
GH	200	5	5	100	45
	100	21	19	90	26
	50	15	12	80	16
Prolactin	200	3	0	0	0
	100	4	0	0	0
Frogs' own anterior pit. lobes		29	29	100	100
Frogs' own brain		29	0	0	0

ard frog pituitary extract used to sensitize the ovaries never by themselves caused ovulation during this period.

DISCUSSION

The effect of growth hormone on amphibian ovulation has been studied hitherto only by Ramaswami (18); this author induced ovulation in *Rana cyanophlyctis in vivo* with injections of 2 pituitary glands of the same species given in combination with a commercial preparation of beef growth hormone. The results of our experiments demonstrate that GH may act directly on the ovary.

The objection might be made that the ovulation induced by GH could be caused by an ICSH contamination; however, biological tests have revealed that the highly purified sheep growth hormone used in these studies contained less than 0.2% of ICSH activity (17). Such a contamination cannot be responsible for the 80% positive response elicited by GH in a concentration of 50 μg. per ml., since a dose of 5 μg. of ICSH per ml. gives only a 30% positive response (Table 2).

Since it is known that growth hormone influences water metabolism (see for example (19)), and also that hypotonic solutions are capable of inducing ovulation *in vitro* (20), the ovulation-inducing capacity of growth hormone noted in these studies might possibly be ascribed to some enhancement of water uptake or retention. This possibility seems to be discounted, however, by observations made by us (21), to the effect that although when ovarian fragments were immersed in either distilled water or 10% Holtfreter's solution alone, the resulting eggs were markedly swollen, no discernible swelling was noted in the eggs from those ovarian fragments that had been immersed in any of the hormone solutions. What relevance these observations may have to the possible mechanism of ovulation, particularly with respect to theories relating to tissue permeability, cannot be determined here.

Our findings with respect to sheep FSH are in agreement with those of Wright and Hisaw (13), who also were unable to induce ovulation *in vitro* in ovaries of *Rana pipiens* with this hormone. Their opinion that FSH sensitized the ovaries, however, was not supported by the results of our experiments carried out with combinations of FSH and ICSH.

The gonadotropic activity of progesterone in amphibians first demonstrated by Zwarenstein (9), with *Xenopus laevis* used as a test animal, was confirmed by the results of our experiments on *Rana pipiens*. In the present experiments, progesterone in a concentration as low as 0.6 μg./ml. was sufficient to cause ovulation *in vitro* (Table 1). As already mentioned, cortisone is also capable of facilitating the process of ovulation *in vitro* in *Rana pipiens* (12). These results suggest that progesterone and related steroids play an important role in the process of amphibian ovulation;

hence, it would be very valuable to investigate which steroids, if any, are present in amphibian ovarian and adrenal tissue.

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THE HEPATIC RESPONSE TO GLUCAGON IN THE UNANESTHETIZED DOG[1]

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ABSTRACT

Intravenous administration of crystalline glucagon .02 mg./kg. was used to study hepatic glycogenolysis and associated events in the unanesthetized dog. An increase in hepatic blood flow and hepatic glucose output regularly occurred following glucagon administration. Approximately 9.6 gm. of glucose were released from the liver during the first hour after a single intravenous injection of glucagon; this represents a net increase over control values of about 6 gm. Depletion of glycogen stores induced by fasting was associated with a decreased or absent response of hepatic blood flow and hepatic glucose output to glucagon. Hepatic uptake of alpha amino nitrogen increased after glucagon-induced glycogenolysis; this increase began promptly and reached a maximum about 45 minutes after glucagon administration. These changes were interperted as reflecting increased gluconeogenesis following depletion of liver glycogen stores.

GLYCOGENOLYSIS may be induced in the liver in a controlled fashion by means of glucagon administration. Accordingly, glucagon has been found to be a useful tool for the study of the metabolic and physiologic events associated with hepatic glycogenolysis (1).

Recently, a method for simultaneous catheterization of splenic artery, portal vein and hepatic vein in a simplified one-stage abdominal operation in the dog has been described (2). Blood samples may be simultaneously obtained from these sites after the animal has recovered from the anesthesia and surgical procedure. By use of this preparation, it has been demonstrated that when glucagon-induced glycogenolysis occurs in the dog there is a simultaneous, marked increase in hepatic blood flow (3).

Using this technique for repeated sampling from the hepatic vessels,

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further studies have been made of the glycogenolysis phenomenon, with particular reference to associated changes in hepatic blood flow in relation to portal and hepatic venous glucose concentrations, plasma osmolarity, and alpha amino nitrogen concentrations.

The direct method of sampling blood or plasma concentration gradients across the liver were combined with improved methods for measuring hepatic blood flow in order to provide quantification of hepatic function in the unanesthetized state. These measurements of regional metabolism may be expressed in terms of rate relationships during a control period and as a function of time after the experimental variable. Thus, hepatic metabolism may be approached in a more direct manner, rather than by inference from blood or plasma concentration changes.

METHODS

Fourteen male and female dogs weighing from 12 to 31 kg. were used in these experiments. Plastic catheters were implanted in the left common hepatic vein, the portal vein, and the splenic artery, according to a previously described technique (2). One to thirteen days after the operation, the events associated with glucagon-induced glycogenolysis were studied after fasting for 18 to 24 hours and without sedation. The dogs, trained to stand in a Pavlov frame, were given a period for equilibration in order that conditions might approach those of a steady state. Blood samples were simultaneously aspirated through arterial, portal and hepatic vein catheters; this procedure was repeated at 10-minute intervals for 3 to 6 times during a control period. Following administration of crystalline glucagon at a dose of .02 mg./kg., samples were obtained at 10- to 15-minute intervals for the first hour, and less frequently thereafter.

Hepatic blood flow (HBF) before and after glucagon administration was measured by bromsulphalein technique of Bradley *et al.* (4), and by a modified bromsulphalein method (5). In two experiments HBF was also measured with an indicator dilution method using Cr^{51} labeled red cells (6). Glucose in whole blood was measured by the Nelson-Somogyi method (7); plasma osmolarity was obtained by the freezing point depression method (Fiske Osmometer); and serum alpha amino nitrogen by the method of Troll and Cannan (8).

Independent measurement of the arterial, portal venous and hepatic venous glucose concentrations allows direct comparison of concentration gradients across certain anatomical areas. In this series, the hepatic venous-portal venous glucose concentration difference is taken, as a first approximation, to represent the total hepatic gradient. It is realized that this gradient is not absolutely indicative of the total hepatic gradient; Blalock and Mason (9) found that the portal vein supplies an average of 80.5% of the total hepatic inflow under resting unanesthetized conditions. If this value is assumed to represent the proportion of portal venous inflow to the total hepatic flow in this series, a calculated total hepatic glucose concentration gradient may be derived:

$$\text{Total Hepatic Glucose Concentration Gradient} = (\text{Glucose}_{\text{H.V.}}\text{-Glusoce}_{\text{P.V.}}) \times .805 + (\text{Glucose}_{\text{H.V.}}\text{-Glucose}_{\text{A.}}) \times .195$$

where $\text{Glucose}_{\text{H.V.}}$, $\text{Glucose}_{\text{P.V.}}$ and $\text{Glucose}_{\text{A.}}$ represent the glucose concentrations in the hepatic venous, portal venous and arterial blood respectively. When this formula is used to calculate the total hepatic gradient, the results of the series are 10% lower than the simple hepatic venous-portal venous gradient in the control period and 7% lower in the period after glucagon. These differences are relatively small and do not alter the final interpretation. Since methods are not presently available for frequent simultaneous measurements of hepatic arterial and portal venous flow under the conditions

of these experiments, we have used the hepatic venous-portal venous gradient as a first approximation of the total hepatic concentration gradient in this study.

RESULTS

1. The Effect of Glucagon on Arterial, Portal and Hepatic Venous Glucose Concentrations

The mean control glucose concentrations of arterial, portal and hepatic venous blood of the entire series were 96, 92 and 100 mg./100 ml. respectively. These levels rose to a maximum of 146, 137 and 162 mg./100 ml., twenty minutes after glucagon administration (Fig. 1). The mean portal-hepatic venous glucose gradient and the standard errors of the mean, illustrated in Figure 1, rose from 8 ± 1.3 mg./100 ml., in the control period, to 25 ± 4 mg./100 ml., twenty minutes after glucagon. The arterial glucose levels closely paralleled the portal levels.

2. Hepatic Blood Flow (HBF) Measurement Before and After Glucagon

a) HBF was measured by a bromsulphalein method in 14 experiments on 12 dogs before and after glucagon administration (Fig. 2). An increase in HBF in each experiment was observed 10 to 20 minutes after glucagon. The maxima of the HBF measurements after glucagon ranged from 141%

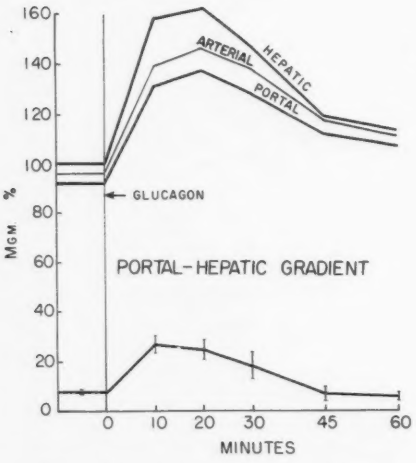

FIG. 1. Mean arterial portal and hepatic venous glucose concentrations for the series during a control period and after glucagon. The lowest line represents the mean portal-hepatic plasma glucose concentration gradients with standard errors of the means.

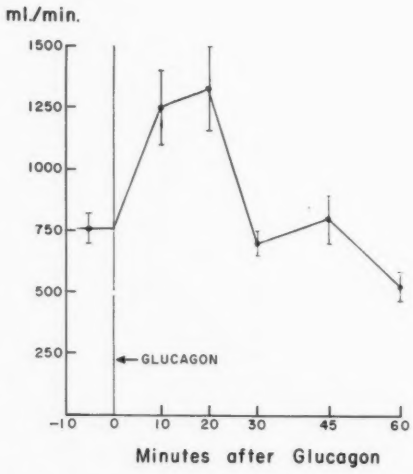

FIG. 2. Hepatic blood flow during a control period and as a function of time after a single intravenous injection of glucagon (indicated by vertical line). Mean and S.E. of 14 experiments.

to 304% of their respective control values. In 5 experiments these peaks occurred at 10 minutes; in 5 other experiments, at 20 minutes after glucagon was given; in the remaining 4 experiments the values at 10 and 20 minutes after glucagon were approximately equal. The mean maximal HBF, irrespective of the period of time after glucagon, was 1731 ml./min., about a twofold increase over the baseline flow of 764 ml./min.

b) Cr^{51} labeled red cells and BSP were used nearly simultaneously as methods of determining hepatic blood flow in 2 dogs before and after glucagon administration. The comparison of these methods is shown in Figure 3. Exact agreement cannot be expected with rapidly changing blood flows; nevertheless, a similar increase was observed with each method.

c) The effect of glucagon administration on HBF was studied again in one dog after 3 days of fasting. The increase in HBF after glucagon was less marked than had previously been obtained in the same animal eight hours postprandially. Another animal was studied after 5 days of fasting and in this case there was no significant increase in HBF following glucagon administration (Fig. 4).

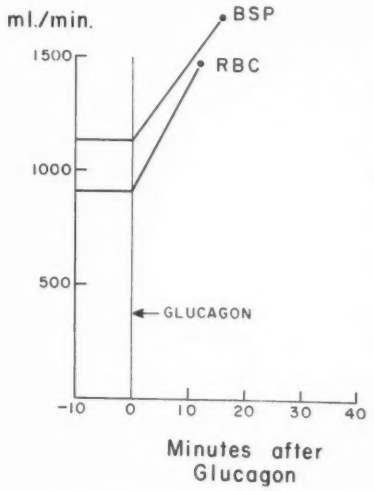

FIG. 3. Measurement of hepatic blood flow with the BSP and Cr^{51} red cell methods. Measurements were taken in sequence rather than simultaneously; both methods show a proportional increase in flow.

3. Hepatic Glucose Output Response to Glucagon

The rate of hepatic glucose output (HGO) in the control period and after administration of glucagon for the 14 experiments is summarized in Figure 5. The variations in values for glucose output at 10 and 20 minutes after the drug are a reflection of the individual variations in the time course of the response. In about half the experiments the maxima were observed at 10 minutes; in the other half, at 20 minutes.

The increased HGO was demonstrable in each experiment. The mean control HGO of the series was 59 ± 10 (S.E.) mg./min. The mean HGO was 335 ± 48 mg./min., 10 minutes after glucagon, and 321 ± 79 mg./min., 20 minutes after glucagon. The mean peak HGO, irrespective of the time interval after glucagon administration, was $446 \pm$ mg./min., which represents about a 7 fold increase over the control rate of HGO.

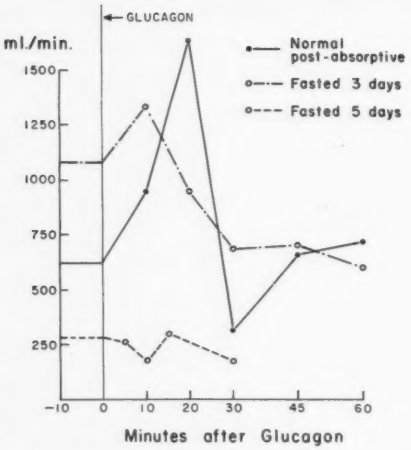

FIG. 4. Hepatic blood flow before and after glucagon administration 18 hours postprandially in a normal animal; in the same animal after a 3-day fast, and in another animal after 5 days of fasting.

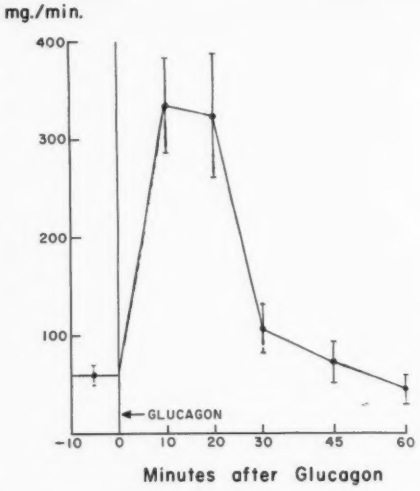

FIG. 5. Hepatic glucose output during a control period and as a function of time after glucagon. Mean and S.E. of 14 experiments.

The effects of depletion produced by 3 and 5 day periods of fasting respectively are shown in Figure 6. A very modest increase in HGO followed glucagon injection in the animal fasted for 3 days, whereas no significant increase was observed after glucagon administration following a 5 day fast.

4. Effect of Glucagon on Portal and Hepatic Venous Plasma Osmolarity

Portal and hepatic venous plasma osmolarity was measured before and after glucagon administration in 4 dogs. The mean values, illustrated in Figure 7, demonstrate an increased hepatic venous plasma osmolarity at 10 and 20 minutes after glucagon. These values closely parallel the corresponding elevations in hepatic glucose output described above.

5. Amino Nitrogen

Plasma alpha amino nitrogen concentrations were measured across the liver during the control period and after glucagon in four normal animals and one animal fasted for 3 days. The data from Dog 5 representative of the series, are shown in Figure 8. In this animal, sampling was carried out for 120-minutes after glucagon injection. There was a significant increase in the hepatic uptake of amino acids. This increase occurred promptly and reached a peak at 60 minutes. Figure 9 shows the hepatic output or uptake of alpha amino nitrogen for the 4 dogs. The mean control value of about 1 mg. alpha amino nitrogen/min. (or .07 mM amino acids/min.) uptake

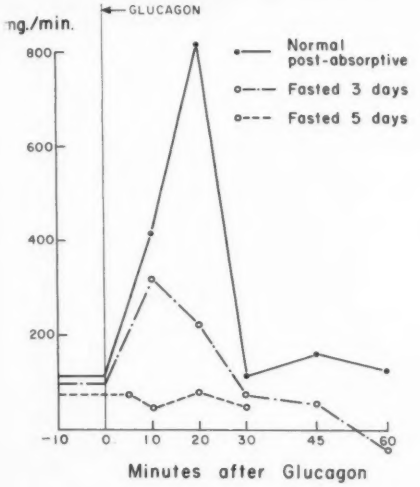

FIG. 6. Hepatic glucose output in the 3 experiments illustrated in Figure 3, on a normal dog, a 3-day fasted dog and a 5-day fasted dog. There is a marked diminution in hepatic glucose output in response to progressive fasting.

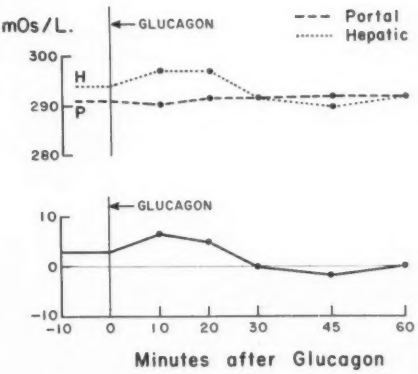

FIG. 7. Portal and hepatic venous plasma osmolarities and transhepatic gradient of osmolarity after glucagon administration. Mean of 4 dogs. Increased hepatic venous osmolarity in response to glucagon roughly parallels increase in hepatic venous glucose concentration.

increased after glucagon administration to a maximum rate of 4.1 mg./min. (0.3 mM/min.) at 45 minutes.

The one animal that had an hepatic output of amino acid nitrogen in the control period demonstrated a rapid reversal in gradient with a fairly marked hepatic uptake after glucagon, reaching a peak at 20 minutes, and returning toward control values thereafter. The animals that were taking up amino acid nitrogen during the control period continued to remove amino acids at increasing rates after glucagon; in these instances, responses were less dramatic, but more prolonged. The results in one dog who was fasted for 3 days are noteworthy. This dog showed an hepatic uptake of 3 mg./min. during the control period; after glucagon this rate of uptake did not increase.

DISCUSSION

The chemistry, physical properties, and structural analysis of glucagon as well as its physiologic or pharmacologic actions have been extensively reviewed (10, 11, 12). Studies employing liver slices have elucidated the mechanism by which glucagon induces hyperglycemia by increasing active phophorylase. (13). The circulating blood or plasma glucose concentra-

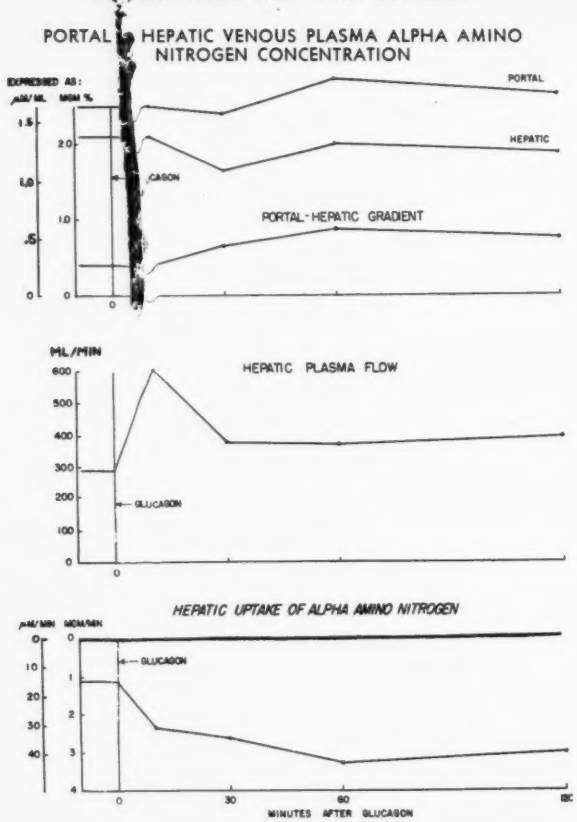

FIG. 8. Portal and hepatic venous plasma α-amino nitrogen concentrations, portal-hepatic gradient, hepatic plasma flow and net hepatic uptake of amino nitrogen of a representative dog in a control period and as a function of time after glucagon administration.

tions and arteriovenous gradients have been studied in man (14, 15). The present research attempts to describe and measure the action of glucagon on *in vivo* hepatic metabolism in the conscious dog.

Simultaneous measurement of hepatic plasma flow and concentration gradients of various substances across the liver permits quantitative assessment of the rates of hepatic function before, during and after glucagon-induced glycogenolysis. The measured hepatic glucose output may be compared with simultaneous increases in arterial, portal and hepatic venous blood glucose concentrations before and after glucagon administration. The mean portal venous glucose level rose from 92 to 137 mg./100 ml., 20 minutes after glucagon, the hepatic venous glucose level from 100 to 16[illegible] mg./100 ml. and the arterial glucose level from 96 to 146 in this period. These increments, 35, 62 and 50 mg./100 ml. respectively, represent 38

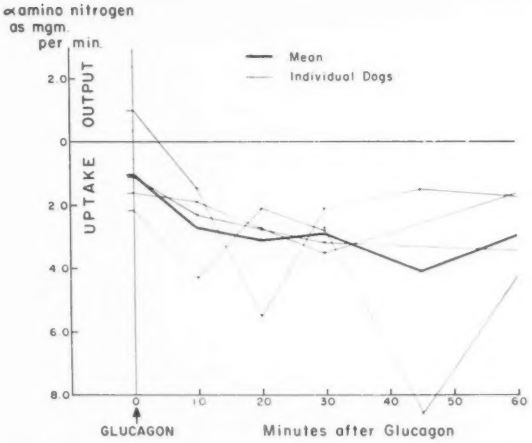

FIG. 9. Net uptake of α-amino nitrogen by the liver after glucagon administration. The heavy line represents the mean values; the fine lines represent values from individual dogs in the series.

62 and 52% increases. Concomitant measurements of the mean hepatic glucose output, by contrast, revealed a rise from 59 mg./min. in the control period to 321 mg./min.; 20 minutes after glucagon, a fivefold increase.

The time relationship shown in the portal venous or arterial glucose concentration curves may also be compared with that of the hepatic glucose output. In all three sites sampled the mean glucose concentrations rise to maxima at 20 minutes after glucagon, and then fell slowly during the ensuring hour toward control levels. Although the rate of HGO also reaches its maximum at 10 to 20 minutes after glucagon, it was found to return to control values at 30 minutes. Since blood glucose levels depend upon hepatic output, redistribution of glucose and peripheral utilization, as well as renal excretion at high levels, neither the rate nor the time relationships of the hepatic glucose output may be inferred solely from the measurement of circulating blood glucose concentrations.

The average amount of glucose released by the liver after glucagon administration was calculated from the area under the time-rate curve (Fig. 4). Approximately 7.5 gm., or a net increase of 5.5 gm. over control values, was released during the first 30 minutes; about 9.6 gm., or a net increase of 6 gm., was released during the first hour after glucagon administration. The mean liver weight in a series of sixteen dogs studied in this laboratory was 2.9% of total body weight; the glycogen content varied from 2 to 4% of the wet liver weight (mean = 2.5%). As a first approximation, therefore, these livers contained about 12 grams of glycogen, the equivalent of 25 mgm. of glucose per gram of wet liver weight. Thus, these calculations suggest that about half of the liver glycogen may be released as a result of a single injection of .02 mg. glycagon/kg. When the

liver was depleted of glycogen by fasting, this glycogenolytic response was modified or absent.

An index of gluconeogenesis (glucogenesis from non-glucose precursors) is provided by measurement of the rate of hepatic alpha amino nitrogen uptake. The rather prompt increase in this rate following glucagon administration suggests that gluconeogenesis begins with the onset of glycogenolysis; maximal amino acid nitrogen uptake, however, was not observed in our series until after subsidence of glycogenolysis. We regard this increased uptake of alpha amino nitrogen as a compensatory reaction to depletion of liver glycogen and not as a primary response to glucagon administration, as has been suggested by Izzo and Glasser (16).

The total amount of alpha amino nitrogen taken up by the liver, estimated from the area under the time-rate curve, averaged 187 mg. of alpha amino nitrogen or 13 mM of amino acids in the first hour after glucagon. Concomitantly, the liver released an average of 9.6 gm. of 53 mM of glucose. Thus, there appears to be a rapid and extensive replenishment of hepatic glycogen stores from plasma amino acids following glycogenolysis.

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INDUCTION OF OVULATION IN THE HEN BY INJECTION OF PROGESTERONE INTO THE BRAIN

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ABSTRACT

Progesterone in a solution of propylene glycol regularly induced premature ovulation in the hen when injected into certain regions of either the hypothalamus or the caudal neostriatum but not in other parts of the forebrain. The effective quantity of hormone is estimated to be no more than 5 μg. Progesterone did not induce ovulation when injected into the pituitary. It is thus indicated that the steroid acts upon certain regions of the brain to cause release of ovulation-inducing hormone.

THERE is considerable indirect evidence, reviewed previously (1), to support the belief that progesterone injected by various systemic routes causes premature ovulation in the hen by acting through a neural mechanism. Two investigations have provided evidence of a direct nature that this is so. Rothchild (2) applied progesterone directly to the surface of the pituitary and found it no more effective in inducing ovulation than when placed upon the sphenoid bone covering the gland or on an exposed area of the thigh. He suggested that progesterone acts through some systemic mechanism apart from the pituitary gland. Ralph and Fraps (1) were able to prevent the occurrence of induced ovulation by placing lesions in the preoptic hypothalamus of the hen within 2 hours following intramuscular injection of a normally-effective dose of progesterone.

This report describes experiments in which small quantities of progesterone were injected directly into various parts of the brain. The results provide further evidence that progesterone probably acts through specific parts of the brain to effect the release of pituitary gonadotrophin for ovulation.

MATERIALS AND METHODS

All experimental animals were White Leghorn hens between 1½ and 2 years of age. They were individually caged in laying batteries with feed and water always available and were under 14 hours light daily (daylight not excluded). Individual records of lay were maintained from 8 A.M. to 4 P.M.

Hens were selected for experiment on the day of lay of the terminal egg of a sequence,

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and were injected between 3:30 and 5:30 P.M. of the same day. The stereotaxic procedures used in making lesions (3) were employed for introducing the hormone into the brain, except that a microinjector[2] with a 26 gauge needle replaced the electrode carrier. Except for five injections directly into the pituitary gland, all were placed bilaterally. Injections into the brain were made 1 mm. from the midline throughout the diencephalon, with a few at 3 mm. In the forebrain, injections were placed, bilaterally, at 1, 3, 5, 7, and 9 mm. from the midline and at 2 mm. intervals both dorso-ventrally and rostro-caudally on the sagittal plane.

In preliminary experiments, aqueous suspensions of progesterone microcrystals[3] and corn oil solutions of the hormone were injected into a total of 17 hens. The aqueous suspension proved difficult to use owing to sedimentation of the crystals and consequent inability to control dosage. Corn oil solutions were somewhat too viscous for easy filling of the microinjector.

In the definitive experiments, progesterone was dissolved in propylene glycol, 5 mg. per ml. A small quantity of sudan black B was used to color the solution. The stain served two purposes: (1) It facilitated the localization of sites of injection during sectioning of the brain and (2), it permitted an approximation of how much of the solution remained at the site of injection, since there was usually some leakage back to the surface following withdrawal of the needle.

A total of 85 hens were injected with the propylene glycol solution. Five μg. of progesterone (0.001 ml. solution) were injected at each site, or 10 μg./hen. It is estimated that no more than half the solution stayed at the site of injection, as some flowed up the needle track and appeared on the surface just as the needle was withdrawn. It is to be emphasized, however, that most of the solution remaining in the brain could be detected visually upon sectioning as a localized colored spot at the actual site of injection; little was seen in the track itself.

The hens were palpated at 8:30 A.M. and 2:30 P.M. during the day following injections to determine whether premature ovulation had or had not occurred (cf. 1). Within 1 to 7 days following injections the birds were sacrificed by intravenous injection of Nembutal, the brains were dissected out and fixed in formalin, and the condition of the ovary was noted. Sections 100 μ thick were cut using a freezing technique and then stained with thionin. When the microinjections were 1 mm. from the midline bilaterally, sagittal sections showing sites of injection on both sides were made, and when more than 1 mm. the brains were sectioned transversely.

Control injections were made with propylene glycol colored with sudan black B but containing no progesterone. Six hens were injected in the preoptic hypothalamus and six in the effective region of the neostriatum. The same conditions and methods were used as for the experimental animals, except that some brains were hand sectioned.

As additional controls, groups of hens from the same flock were injected intramuscularly (breast muscle) with the propylene glycol solution of progesterone at doses of 10, 50, and 100 μg.

RESULTS

Injections induced premature ovulation when placed 1 mm. bilaterally either in the anterior and ventral hypothalamus or in the caudal neostriatum (Fig. 1). In the hypothalamus, the more anterior and dorsal effective loci are within the preoptic paraventricular nucleus, while the one

[2] Baltimore Instrument Company, Baltimore, Maryland.

[3] We wish to thank the Upjohn Company, Kalamazoo, Michigan, for a supply o progesterone in aqueous suspension.

xtending along the dorso-caudal surface of the optic chiasma are in the nterior hypophyseal tract. The four most posterior effective sites lie vithin the tubero-mammillary region.

One premature ovulation occurred after bilateral injections 5 mm. from he midline opposite the effective region in the caudal neostriatum. All ites of response, except this one, are shown in Figure 1. Ovulation was in- uced in the hypothalamus only when the tip of the needle entered the entral border (Fig. 1). When the tip of the needle was in regions dorsal to

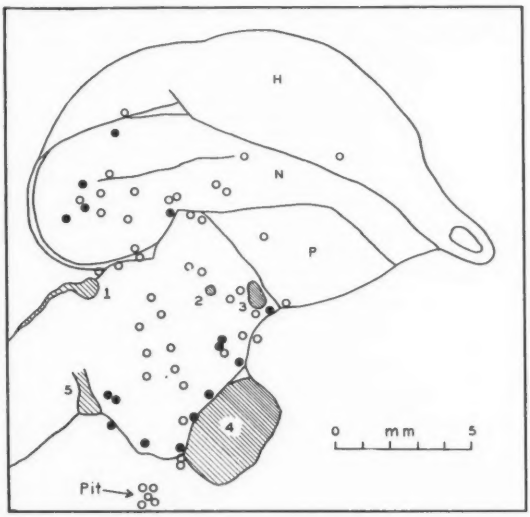

FIG. 1. A parasagittal view of the hen's diencephalon and forebrain, showing the sites of injections of progesterone made bilaterally at 1 mm. Solid circles, induced premature ovulation; open circles, no premature ovulation; 1, posterior commissure; 2, anterior commissure; 3, tractus septo-mesencephalicus; 4, optic chiasma; 5, oculomotor nerve; Pit, pituitary; H, hyperstriatum; N, neostriatum; P, paleostriatum.

this ventral-most border, and thus along the tracks of injections at or near the border, ovulation was not induced. Despite the leakage observed on withdrawal of the needle, these relationships make it reasonably certain that the effect of injection was concentrated at the point of actual delivery of the hormone.

Results from hens injected with progesterone in aqueous suspension or in corn oil solution support these findings, in that those ovulating prematurely (6 out of 17) had been injected in one of the two effective regions just described.

It is of particular interest that the five birds in which progesterone was injected directly into the pituitary gland (confirmed by histological examination or by hand sectioning) failed to ovulate prematurely.

None of the twelve hens receiving control injections of propylene glycol

responded by ovulating prematurely. Of those hens injected intramuscularly with the progesterone solution, none out of 9 responded at 10 μg., but 6 out of 7 did so at 50 μg. and 4 out of 5 at 100 μg.

DISCUSSION

It would appear that progesterone exerts its stimulatory effects upon a number of structures in the hypothalamus and an extensive region of the caudal forebrain. Even though the injections were placed in a systematic manner throughout the forebrain and median diencephalon, it is possible that other progesterone-sensitive regions may exist in the brain but were not found in this survey. Injections at more closely spaced intervals will have to be made to explore such a possibility.

The demonstration that the paraventricular nucleus and the anterior hypophyseal tract are effective sites for stimulation may be correlated with the finding that lesions placed in the same region within 2 hours after the systemic injection of progesterone prevent the expected ovulation (1). At the same time it is interesting that such lesions, destroying only a portion of the progesterone-sensitive structures in the hypothalamus, are able in some way to prevent ovulation. The question is raised whether a lesion in any of the progesterone-sensitive structures could effectively prevent induced ovulation. Preliminary results (unpublished) indicate that lesions about 2 mm. in diameter in the neostriatum are ineffective in preventing progesterone-induced ovulation. The tubero-mammillary region remains to be investigated.

The failure of progesterone to induce ovulation when injected into the pituitary confirms the conclusion of Rothchild (2) that progesterone does not cause release of ovulation-inducing hormone by direct stimulation of that endocrine gland.

Although no attempt was made to estimate the minimal dose for inducing ovulation, about 5 μg. of progesterone was an effective quantity when placed at a proper site in the brain. This may be compared with the results of intra-muscular injections, where 10 μg. were ineffective but 50 and 100 μg. induced ovulation. Thus, the effective dose when administered intramuscularly must be at least twice and perhaps 10 times that needed when injected into responsive sites of the brain to induce ovulation regularly. It is of some interest that the hens used in the present experiments appeared to be more sensitive to subcutaneously injected progesterone in propylene glycol than were the hens used by Fraps and Dury (4) to progesterone in corn oil.

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PITUITARY GONADOTROPHINS IN THE EWE AT DIFFERENT STAGES OF THE ESTROUS CYCLE

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ABSTRACT

The content of FSH, LH, and LTH in ewe pituitaries has been determined at the following stages of the estrous cycle: 4 hours, 35 hours, 10 days, and 15 days after first acceptance of the ram. Pituitary FSH and LH decreased significantly between 4 hours and 35 hours after first acceptance, and then returned to proestrus levels by the fifteenth day of the cycle. Pituitary LTH content did not vary significantly during the estrous cycle with the assay techniques employed. It is suggested that FSH plays an active role in the induction of ovulation in the ewe and that the assumption of a specific release of LH may not be valid for this species.

THE postulate that follicle stimulating hormone (FSH) and luteinizing hormone (LH) are needed in sequence for certain of the ovarian changes to proceed suggests that different concentrations are necessary at different times of the estrous rhythm. For this reason, studies of the variations in gonadotrophic activity of the anterior pituitary as associated with different stages of the estrous cycle are important.

Some studies of total gonadotrophic activity of the pituitary have been made. Thus, a reduction in activity at estrus and during the luteal phase has been reported for the sow (1, 2), guinea pig (3), rat (4), and ewe (5).

Qualitative differences in the response of the assay animal has suggested that pituitary FSH and LH activities may vary independently during the cycle (3, 4). Changes in pituitary luteotrophic hormone (LTH) have not been reported.

Preliminary observations previously reported by us (6) suggested that the anterior pituitary content of LH and LTH did not vary significantly during the estrous cycle of the ewe. In contrast, a marked reduction of FSH had occurred between the fourth and thirty-fifth hour after onset of estrus.

The present studies were conducted to further investigate changes in the pituitary gonadotrophins during the cycle and to elucidate the pos-

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[2] The data contained in this paper is part of a thesis submitted by the senior author to the Graduate School of the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

sible importance of FSH in the ovulating mechanism. In certain respects, the present study was better controlled than the earlier one; the ewes were more uniform in age and the glands from all intervals were assayed simultaneously.

METHODS

Forty-three cross-bred, white faced ewes, ranging in age from 2 to 4 years, were tested daily for estrus with an "aproned" or vasectomized ram. The time of estrus was designated as the day when the ewe first accepted the male, and the length of the cycle was the interval between the occurrence of each estrus. After the regularity of estrous cycles was established, the ewes were sacrificed so that ten ewes were included in each of the following stages: 4 hours and 35 hours after first acceptance of the male, the tenth and fifteenth day post-estrus. Three ewes were sacrificed on the fifth day post-estrus. The moment of onset of sexual receptivity, for those ewes in the 4 and 35 hour groups, was determined by placing a vasectomized ram with the ewes and observing continuously for 24 hours a day.

Animals were sacrificed by exsanguination. The pituitary gland was removed as quickly as possible, weighed, frozen with dry ice, and the posterior lobe removed. The anterior lobes were lyophilized and stored in the dry state until assayed. The reproductive tract of each ewe was removed and macroscopic observations recorded. The ovaries and adrenals as well as pieces of the uterus, cervix, and vagina were fixed in 10% formalin. The ovaries were bisected through the largest follicle and corpus luteum to measure the diameters of these structures. All tissues were sectioned at 10 μ and stained with hematoxylin and eosin for histologic examination.

The ten anterior pituitaries representing each stage, except the 5-day group, were randomly paired, thus providing sufficient material in each of the five samples to evaluate the content of the three gonadotrophins. Since only three ewes were involved in the 5-day group, their pituitaries were tested individually for FSH and LH only.

FSH determinations were based on the augmentation reaction with human chorionic gonadotrophin (HCG) described by Steelman and Pohley (7). Twenty-one to twenty-two day old intact, female Sprague-Dawley rats were injected subcutaneously once daily over a period of three days with a saline suspension of pituitary powder. In all cases 20 I.U. of HCG (Antuitrin-S)[3] served as the augmenting dose. Each rat received a total of 16 mg. of dried anterior pituitary combined with the HCG. Control rats received HCG alone. At necropsy, 72 hours after the first injection, the ovaries were dissected free of surrounding tissue and weighed to the nearest 0.2 mg. FSH activity was indicated by the increase in ovarian weight over that of the HCG controls.

LH assays were based on the method described by Greep, *et al.* (8). Male Sprague-Dawley rats were hypophysectomized at 21 to 22 days of age. Beginning on the second post-operative day, they were injected intraperitoneally twice daily for four days with a total of 2 mg. of dried anterior lobe powder suspended in saline. At necropsy, 96 hours after the first injection, the ventral prostate was removed and weighed to the nearest 0.2 mg.

LTH was estimated by the crop gland response in market age Silver King squabs. A total of 8 mg. of anterior pituitary was administered by subcutaneous injections given once daily over a period of four days. On the fifth day, the stimulated portions of the crop sacs were dissected free and weighed to the nearest 0.01 gm. (9).

Ewe pituitaries from each of the four intervals of the cycle were tested simultaneously

[3] Parke Davis Company.

n each group of assay animals used. An analysis of variance with two-way classification vas made. Thus, variation in response arising from changes in hormone activity as associated with the stage of the cycle was separated from variation due to differences in ensitivity between groups of assay animals.

RESULTS AND DISCUSSION

Cycle Length and Ovarian Changes. To properly interpret the findings on ituitary gonadotrophin content, it is essential to have information on he length of the cycle and ovarian changes in the ewes employed. In this tudy, the average cycle length of 18 days is slightly greater than that eported by most investigators. Since the majority of cycles fell between 4 and 19 days, it is possible that those recorded as 28–38 days are double ntervals in which behavioral heat was not observed at the expected time. f this assumption is correct, the average would be somewhat closer to 17 days in length.

No instance of ovulation was seen in the ovaries of those animals slaughtered at four hours after first acceptance. By contrast, recent ovulation had occurred in all animals sacrificed at 35 hours, an observation in agreement with the report of Cole and Miller (10) that ovulation occurs about 30 hours after onset of heat in this species. Macroscopically the corpora lutea observed at 5, 10, and 15 days as well as at four hours of the succeeding cycle appeared as healthy functional structures. At 35 hours these structures, from the preceding cycle, were less vascular as indicated by their anemic appearance. The diameter of the corpus luteum reaches its maximum size about the tenth day of the cycle. Follicular growth, on the other hand, appears to be continuous throughout most of the cycle.

Histologically, the freshly ruptured follicle (at 35 hours) is lined with a deeply folded granulosa with little or no sign of luteinization at this time. After five days the corpus luteum is well formed, but luteinization of the granulosa cells is not as yet complete. By the tenth day post-estrus vacuolization of the luteal cells and fibroblast infiltration indicate that regressive processes have begun. These changes progress slowly up to four hours after onset of estrus of the succeeding cycle. In agreement with the findings of Quinlan and Mare (11), marked regression of the corpus luteum from the preceding cycle has occurred by the thirty-fifth hour after onset of estrus. These changes include extensive fibroblast infiltration, pycnotic nuclei, and reduced cell size.

Histological examination of the uterus, vagina, cervix, and adrenal gland did not reveal marked changes associated with the cycle nor did pituitary and adrenal gland weights vary significantly.

LTH Content. In accord with our earlier findings, the lactogenic hormone content of sheep pituitaries did not differ significantly among the various stages of the estrous cycle. The average weight of crop sacs in mg.

for each stage of the cycle was as follows: 4 hours, 3.0; 35 hours, 3.9; 10 days, 3.8; and 15 days, 3.3. For the untreated controls, the average crop sac weight was 1.4 mg.

On the basis of diameter measurements and histological study, rapid regression of the corpus luteum does not occur until after ovulation of the succeeding cycle at a time when the new corpus luteum is forming. For this reason, appreciable changes in pituitary LTH content would not necessarily be expected.

LH Content. A statistically significant drop occurred between the fourth

TABLE 1. GONADOTROPHIC HORMONE CONTENT OF SHEEP PITUITARIES AT VARIOUS STATES OF THE ESTROUS CYCLE

(Each per cent increase refers to the response obtained by a pair of pituitaries)

	Stage of cycle				
	4 hrs.*	35 hrs.*	5 days†	10 days	15 days
FSH	20(6)‡	13(6)	1(5)	27(6)	35(6)
Per cent increase in ovarian	9(6)	−2(6)	−2(6)	12(6)	29(6)
weight over HCG—controls	36(6)	−6(5)	4(5)	18(6)	11(6)
	41(6)	20(6)		34(6)	46(6)
	67(6)	54(6)		69(6)	61(6)
Mean ± S.E.	35 ± 10	16 ± 11	1 ± 2	32 ± 10	36 ± 8
LH	224(6)	157(6)	191(5)	171(6)	232(6)
Per cent increase in ventral	220(4)	89(5)	77(4)	117(5)	114(6)
prostate weight over uninjected controls	237(4)	68(5)	272(5)	117(5)	148(5)
	288(6)	141(6)		206(4)	211(5)
	301(5)	240(5)		191(5)	252(5)
Mean ± S.E.	254 ± 17	139 ± 30	180 ± 57	160 ± 19	192 ± 26

* Time from onset of estrus.

† These values represent responses from individual pituitaries and thus were not included in analysis.

‡ Figures in parentheses refer to number of rats used per assay.

and thirty-fifth hour followed by an increase as the cycle advanced (Table 1).

A drop in LH content in the immediate post-ovulatory period fits well with the present widely accepted concept of LH release as the stimulus for ovulation. Lane and Hisaw (4) found that pituitary LH activity was least in adult rats during diestrus and greatest during estrus and suggested that LH was the "ovulating" hormone. Our results likewise suggest that LH release is involved with release of the egg.

In our previously reported work (6), we did not observe the drop in LH content of the pituitary at the thirty-fifth hour; however, glands from the several intervals were not assayed simultaneously, and thus we have greater confidence in the results obtained in the present experiment. It is impossible by present procedures to estimate the circulating LH activity of a cycling animal beyond the observation that luteinization has occurred. Thus, slight changes in systemic levels of this hormone independent

of pituitary content may have occurred in the earlier trials. In the guinea pig, the amount of LH required to cause luteinization appears to be satisfied by the amount necessary for ovulation (12) and in the hypophysectomized female rat small amounts of LH are sufficient to initiate luteinization when administered with large doses of FSH (13, 14).

FSH Content. In both the earlier experiment (6) and in the present one, the changes in pituitary content of FSH throughout the cycle were consistent. A significant decrease was seen between the fourth and thirty-fifth hour after onset of heat. The level remained low until the fifth day of the cycle after which it increased rapidly and remained high until the next ovulatory period (Table 1).

It is of interest to compare the changes in pituitary FSH with the rate of growth of the follicle. Diameters of the largest follicles at different stages of the estrous cycle indicate appreciable follicular growth between the periods of 35 hours and 5 days. In this period, the content of FSH is lowest. After the fifth day, follicular growth rate is constant up to 15 days and pituitary FSH values are increasing. There is no change in follicular size between day fifteen and four hours after onset of heat of the succeeding cycle. Pituitary FSH activity, likewise, is the same for these two periods.

The follicular diameter measurements suggest that FSH is being secreted continuously by the pituitary from the end of heat to about day fifteen. Thus, the increase in pituitary content of this hormone during the follicular phase would suggest synthesis and storage in excess of secretion. Since both secretory rate and synthesis appeared to have plateaued by day fifteen, a sudden release must explain the drop between the fourth and thirty-fifth hour after onset of estrus.

Constant growth rate of the follicle from the end of one heat period to the onset of the next heat has been observed in the guinea pig (15) and in the rat (16). In these investigations, a marked increase in growth rate from the onset of estrus until ovulation (about 10 hours in both species) was also reported. A preovulatory swelling has been observed in the rabbit (17) and in the ewe (18). The marked drop in pituitary content of FSH between the fourth and thirty-fifth hour associated with the pre-ovulatory swelling supports the concept of rapid release of this hormone at this time.

Nalbandov (19) cites evidence to suggest that in the normal cycling, spontaneously ovulating female, pituitary content of gonadotrophin reflects systemic levels. On the other hand, there is evidence to suggest that the gland content may not reflect the secretory rate under certain conditions. In the anestrous ewe (5), pituitary content is high but the ovaries are relatively inactive. The decreased gonadotrophin content of rabbit pituitaries which occurs 24 hours post-coitus probably represents a rapid release (20), as does the decreased content of pituitary gonadotrophin at the time of estrus in spontaneously ovulating species (1); (2); (3); (4); and (5).

When follicles are sufficiently mature beforehand, ovulation can be readily induced with gonadotrophins rich in follicle stimulating activity (21). Foster and Hisaw (22), using "purified" preparations of FSH and LH, found that a mixture of the two was more effective than either one alone for inducing ovulation in the anestrous cat. The optimum response with their preparations was obtained with a mixture of 50 parts FSH and 1 part LH.

Figure 1 shows clearly the immediate post-ovulatory drop in both FSH and LH. Furthermore, the changes in the content throughout the cycle follow a similar pattern for both hormones. It would be possible, but purely speculative, to interpret these findings as indicating the secretion of a protein complex having both FSH and LH activities. Such a view gains support from the recent work of Rigas, *et al.* (23) in which electrophoretic separation of highly purified human pituitary or urinary gonadotrophin failed to confine FSH and LH activities to separate components.

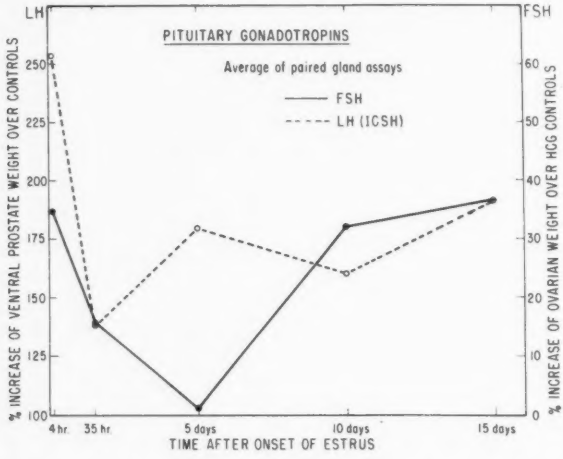

FIG. 1. Pituitary content of LH and FSH hormones during the estrous cycle.

Nevertheless, qualitative changes have been reported in pituitary gonadotrophins (3, 4) as well as in human urinary gonadotrophins (24, 25) throughout the cycle. These observations, of course, are not without their counter evidence (26, 27). Such data as are available do not permit an interpretation of whether there are two hormones involved or one protein complex with two prosthetic groups. Either of these explanations could conceivably permit shifts in the FSH:LH ratio.

Whether or not these activities exist as one or two entities cannot be settled by this study. However, our results clearly demonstrate that both FSH and LH activities are implicated in the induction of ovulation in the ewe. Perhaps more important than slight alterations in the ratio of

FSH:LH liberated, as the stimulus for ovulations, is the rather rapid release of gonadotrophins. Thus, the assumption by many of LH as the *ovulating hormone* may not be valid.

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GLUCOSE AND ACETATE METABOLISM AND LIPOGENESIS IN MAMMARY GLANDS OF HYPOPHYSECTOMIZED RATS IN WHICH LACTATION WAS HORMONALLY INDUCED[1]

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ABSTRACT

Rats which had been hypophysectomized in midpregnancy were injected daily during the postpartum period with either a) mammotrophin, Δ^1-hydrocortisone acetate, and somatotrophin, or b) mammotrophin and Δ^1-hydrocortisone acetate or c) Δ^1-hydrocortisone acetate and somatotrophin or d) one of these hormones alone. The pattern of glucose and acetate metabolism in slices of their mammary glands was evaluated by the following criteria: 1) the extent and relative conversion of glucose carbons 1, 2, and 6 to CO_2 and fatty acids; 2) the relative participation of the Embden-Meyerhof and the hexosemonophosphate oxidative pathways of glycolysis in the conversion of glucose to fatty acids; and 3) the stimulating effect of glucose on lipogenesis from acetate.

Lactation was established and maintained in rats injected with hormones listed under a) and b) above. The metabolic behavior of their mammary glands was identical in all respects with that observed in mammary tissue of normal lactating rats.

A metabolic feature of the hormonally-maintained, lactating mammary glands of the hypophysectomized rats was the participation of a non-Embden-Meyerhof pathway in the conversion of glucose to fatty acids. The appearance of this pathway seemed to be related to milk formation and secretion and not to gland growth and development.

IT HAS been known for many years that hypophysectomy abolishes milk secretion (1), and it has been shown more recently that lactation can be induced in the hypophysectomized rat by administration of various combinations of adenohypophyseal and adrenocortical hormones (2, 3). The effects of these hormones on the histology and lactational performance of the mammary gland have been studied (4–7), but their actions on the metabolism of the gland have not so far been reported. The present report deals with the metabolic behavior of mammary glands of rats that were hypophysectomized in mid-pregnancy, and injected with one or more of the following hormones, beginning on the day of parturition: mammotrophin (lactogenic hormone), somatotrophin (growth hormone), and Δ^1-hydrocortisone (prednisolone). The mammary glands of three groups of such rats were studied. In one, milk secretion was successfully maintained

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by daily hormone treatments during the postpartum period; in the second group, the hormonal treatments failed to establish lactation; in the third, lactation was first maintained by daily postpartum hormonal injections and then arrested by discontinuation of these treatments.

EXPERIMENTAL

Rats and Their Treatment—Rats of the Long-Evans strain were hypophysectomized by the parapharyngeal route on the eleventh day of their first pregnancy, and hormone injections were begun on the day of parturition (Table 1). The advantages of performing hypophysectomy in the middle of pregnancy rather than during lactation have been pointed out by Bintarningsih *et al.* (3). At delivery, each mother was supplied with either six of her own or six foster, new-born pups, and these or other new pups were kept with her for the duration of the experiment. When lactation was not induced, fresh new pups were supplied to the mother daily. Lactational performance was judged by the weight increment of the litter during the last five days of the experiment. The rats were fed, before as well as after parturition, a stock diet I of natural foodstuffs, the composition of which has been described elsewhere (8).

The mothers were killed by cervical fracture on the day after the last of 10 daily hormone injections, and parts of their mammary glands were quickly excised and placed in ice-cold, oxygenated Krebs-Henseleit buffer (9). The remaining glands were used for

TABLE 1. HISTORY OF HYPOPHYSECTOMIZED RATS AND THEIR HORMONAL TREATMENT

Rats of the Long-Evans strain were hypophysectomized on the eleventh day of pregnancy, and hormone injections were begun on the day of parturition. All rats were injected subcutaneously with one or more of the hormones daily for 10 days. All rats except nos. 18 and 19 were killed on the day after the last hormone injection (i.e., eleventh day postpartum). Rats 18 and 19 were killed 4 days after they received their last hormone injection (i.e., 14th day postpartum).

Rat	Body weight		Hormones injected			Gross development of mammary gland*	Lactation†	Growth per pup‡
	At parturition	When killed	Mammotrophin	Δ^1-hydrocortisone acetate	Somatotrophin			
	gm.	gm.	mg. per day	mg. per day	mg. per day			gm. per day
1	196	199	5	0.4	1.0	++++	L	1.3
2	195	187	5	0.4	1.0	++++	L	1.4
3	212	213	5	0.4	1.0	++++	L	0.9
4	219	210	10	0.4	0	+++	L	1.4
5	214	207	10	0.4	0	+++	L	1.6
7	190	188	2.5	0.4	1.0	++++	L	1.4
9	185	187	2.5	0.4	1.0	++++	L	1.2
11	190	195	2.5	0	0	+	NL	0
12	217	227	2.5	0	0	+	NL	0
13		128	0	0.4	0	+	NL	0
14	158	145	0	0.4	0	+	NL	0
15	178	187	0	0.4	1.0	++	NL	0
16	211	211	0	0.4	1.0	++	NL	0
17		296	0	0	1.0	+	NL	0
18	210	204	5	0.4	0	+++	§	
19	199	199	5	0.4	0	+++	§	

* Rated on an arbitrary scale of 0 to ++++, 0 representing gland regression to the duct stage and ++++ the fully developed gland.

† L = lactation, and refers not only to milk secretion but also to delivery as shown by its detection in the stomachs of the sucklings. NL = not lactating.

‡ During last five days before the mother was killed.

§ See text and footnote (*) in Table 4.

microscopic examination. The complete absence of the pituitary glands in all rats was confirmed at necropsy.

We are indebted to Dr. W. R. Lyons and Miss R. E. Johnson for the preparation of the rats. The source and purity of the hormones used in this study, and the lactational and nursing behavior of the rats have been reported by Bintarningsih *et al.* (3).

Substrates—Glucose-1-C^{14} and glucose-2-C^{14} were purchased from the National Bureau of Standards. Glucose-6-C^{14} and acetate-2-C^{14} were prepared in this laboratory. The labeled hexoses used in this study were shown to be chromatographically pure and to contain the isotope exclusively in the designated position (10). The acetate was added to the incubation medium as the sodium salt.

Incubation Procedure—Slices were prepared freehand with the aid of a thin razor blade, washed in four successive portions of cold, oxygenated Krebs-Henseleit bicarbonate buffer to remove as much preformed milk as possible, and gently blotted with filter paper. 500-mg. portions were incubated as described in Table 2.

Analytical Procedure—The collection and assay of $C^{14}O_2$, the isolation of the non-volatile fatty acids, and determination of their C^{14} content were carried out as described earlier (11).

RESULTS

Experiments with Mammary Glands of Hypophysectomized Rats in which Lactation Was Successfully Maintained by Daily Postpartum Hormone Injections

Five rats (1–3, 7, and 9) were injected with mammotrophin, somatotrophin, and Δ^1-hydrocortisone acetate. Rats 4 and 5 received only mammotrophin and Δ^1-hydrocortisone acetate. The state of development of the mammary glands at the time they were excised for study is given in column 7 of Table 1. Milk was expressible from the glands of all seven rats, and each of the pups fostered by these rats gained from 0.9 to 1.6 gm. daily.[2] The glands of these rats were similar in gross and microscopic appearance to those of normal lactating rats.

The metabolism of the mammary glands of these seven hypophysectomized rats is shown in Table 2.

$C^{14}O_2$ Recoveries from Glucose-1-C^{14}, -2-C^{14}, and -6-C^{14}—In a series of experiments carried out with slices prepared from the mammary glands of seven *normal* lactating rats, the $C^{14}O_2$ recoveries ranged from 22.5 to 45.3 per cent for glucose-1-C^{14}, from 12.5 to 19.8 per cent for glucose-2-C^{14}, and from 1.1 to 2.6 per cent for glucose-6-C^{14}; the incubation conditions were the same as those described here. The $C^{14}O_2$ values observed with the mammary glands of the lactating, hypophysectomized rats (Table 2) were well within the range of values observed with normal lactating rats. Furthermore, the relative amounts of $C^{14}O_2$ derived from the three differently labeled glucoses were the same for normal rats and for the hormonally-treated, hypophysectomized rats. Thus, in all cases glucose-1-C^{14} yielded the highest $C^{14}O_2$ values, and glucose-6-C^{14} the lowest.

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[2] The new-born pup of a naturally lactating, Long-Evans rat gains about 2 gm. per day.

TABLE 2. METABOLIC ACTIVITY OF MAMMARY GLAND SLICES PREPARED FROM RATS HYPOPHYSECTOMIZED AT MIDPREGNANCY AND IN WHICH LACTATION WAS SUCCESSFULLY MAINTAINED BY HORMONE TREATMENTS

For history of rats and details of hormone administrations see Table 1. 500 mg. of slices were incubated for 3 hours at 37° in a medium consisting of 4.5 ml. of Krebs-Henseleit bicarbonate buffer at pH 7.4 and 50 μmoles of the labeled substrate† in 0.5 ml. of saline. In the experiments with unlabeled glucose, 50 μmoles of the hexose in 0.1 ml. of saline were added to 4.4 ml. of the bicarbonate buffer. The final volume in all cases was 5.0 ml. The gas phase was 95 per cent O_2 and 5 per cent CO_2. The values recorded are averages of two closely agreeing, separate determinations.

Rat No.	Hormones injected			Substrate		Per cent of C^{14} recovered as:		Per cent of fatty acid-C^{14} derived from added glucose via:*	
	Mammotrophin	Δ^1-hydrocortisone acetate	Somatotrophin	Labeled†	Unlabeled	CO_2	Fatty acids	Embden-Meyerhof pathway	Hexosemonophosphate oxidative pathway
1	Yes	Yes	Yes	Glucose-1-C^{14}	None	46.4	14.6		
				Glucose-2-C^{14}	None	26.7	11.4		
				Glucose-6-C^{14}	None	2.1	28.2	52	48
				Acetate-2-C^{14}	None	2.6	0.6		
				Acetate-2-C^{14}	Glucose	3.2	52.9		
2	Yes	Yes	Yes	Glucose-1-C^{14}	None	26.2	5.7		
				Glucose-2-C^{14}	None	23.4	10.3		
				Glucose-6-C^{14}	None	2.9	15.3	37	63
				Acetate-2-C^{14}	None	15.9	0.5		
				Acetate-2-C^{14}	Glucose	8.8	52.8		
3	Yes	Yes	Yes	Glucose-1-C^{14}	None	41.5	12.3		
				Glucose-2-C^{14}	None	23.7	17.9		
				Glucose-6-C^{14}	None	2.2	24.6		
				Acetate-2-C^{14}	None	1.8	0.5	50	50
				Acetate-2-C^{14}	Glucose	2.6	45.6		
4	Yes	Yes	None	Glucose-1-C^{14}	None	28.5	7.8		
				Glucose-2-C^{14}	None	16.4	14.0		
				Glucose-6-C^{14}	None	1.7	16.0	49	51
				Acetate-2-C^{14}	None	10.8	0.7		
				Acetate-2-C^{14}	Glucose	2.2	27.2		
5	Yes	Yes	None	Glucose-1-C^{14}	None	17.1	5.6		
				Glucose-2-C^{14}	None	9.4	15.6		
				Glucose-6-C^{14}	None	1.1	16.0	35	65
				Acetate-2-C^{14}	None	4.6	0.8		
				Acetate-2-C^{14}	Glucose	2.9	45.7		
7	Yes	Yes	Yes	Glucose-1-C^{14}	None	20.4	5.1		
				Glucose-2-C^{14}	None	12.5	11.2		
				Glucose-6-C^{14}	None	1.2	13.1	39	61
				Acetate-2-C^{14}	None	6.7	1.0		
				Acetate-2-C^{14}	Glucose	3.1	22.4		
9	Yes	Yes	Yes	Glucose-1-C^{14}	None	41.9	10.4		
				Glucose-2-C^{14}	None	19.8	14.9		
				Glucose-6-C^{14}	None	2.1	18.3	57	43
				Acetate-2-C^{14}	None	12.3	1.4		
				Acetate-2-C^{14}	Glucose	5.2	55.5		

* Calculated as follows: value for hexosemonophosphate oxidative pathway

$$= 100 \times \frac{\text{fatty acid-}^{14}\text{ recoveries from glucose-6-}C^{14}\text{ minus fatty acid-}C^{14}\text{ recoveries from glucose-1-}C^{14}}{\text{fatty acid-}C^{14}\text{ recoveries from glucose-6-}C^{14}}.$$

The difference between the value obtained from the above formula and 100 gives the corresponding value for the Embden Meyerhof pathway.

† 1.4 to 1.8 $\times 10^6$ counts per minute of C^{14} per flask.

The similarity in the metabolic behavior of the two groups of mammary glands, those excised from normal lactating rats and those from the hormonally-treated, hypophysectomized, lactating rats, is further brought out by the $C^{14}O_2$ ratios shown in the first four lines of Table 3.

Fatty Acid-C^{14} Recoveries from Glucose-1-C^{14}, -2-C^{14}, and -6-C^{14}—The range of values for fatty acid-C^{14} recoveries observed with mammary gland slices prepared from seven lactating *normal* rats was: 5.2 to 18.6 per cent for glucose-1-C^{14}; 5.9 to 28.8 per cent for glucose-2-C^{14}; and 12.9 to 36.7

per cent for glucose-6-C^{14}. Table 2 shows that the incorporation of these glucose carbons into fatty acids by the mammary tissues of the hormonally-treated, lactating, hypophysectomized rats is within the normal range. The relative conversion of these three carbons to fatty acids was also similar for the mammary glands of the two groups of rats.

Stimulating Effect of Glucose on the Conversion of Acetate to Fatty Acids—A 20- to 50-fold increase in lipogenesis from acetate was observed upon the addition of glucose to the incubation medium in experiments with normal lactating mammary slices. The glands of the hypophysectomized rats in which lactation was maintained by hormonal treatments showed stimulations that ranged from 26- to 100-fold (Table 2).

Calculation of the Relative Participation of the Embden-Meyerhof and the Hexosemonophosphate Oxidative Pathways in the Conversion of Glucose to Fatty Acids by Mammary Glands—The relative participation of the Embden-Meyerhof and hexosemonophosphate oxidative pathways[3] in the conversion of glucose to fatty acids was calculated from the yields of C^{14}-fatty acids observed in the experiments with glucose-1-C^{14} and -6-C^{14}, and the values are given in the last two columns of Table 2. The method of calculation as well as the principles underlying this calculation have been dealt with elsewhere (11, 12). In the mammary glands obtained from lactating, hypophysectomized rats, a considerable portion (43–65%) of the glucose-derived fatty acids arose via the hexosemonophosphate oxidative pathway. This range is similar to that observed with the mammary tissues of normal lactating rats (11, 12, 14).

Experiments with Mammary Glands of Hypophysectomized Rats in which Lactation Was Not Maintained by Daily Postpartum Hormone Injections

Rats 11–14 and 17 received postpartum injections of only one hormone, i.e., mammotrophin or Δ^1-hydrocortisone acetate or somatotrophin. Two other rats (15 and 16) were injected with both Δ^1-hydrocortisone acetate and somatotrophin. No milk was expressible from the glands of rats 11–17 after parturition, and no weight gain occurred in any of their foster-young. Examination of stained specimens of these glands revealed small amounts of undischarged and unresorbed secretory material in areas of the glands showing only partial maintenance of the alveoli.

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[3] Various terms have been used to describe this alternative scheme of glucose utilization. Dickens, in a recent review (13), referred to it as the hexosemonophosphate shunt, and this name was used in our earlier reports on mammary gland and liver (10, 11, 14, 15). Phosphogluconate oxidation was used in a later paper on the liver (16). Warburg-Lipmann-Dickens (17), direct oxidative (18), hexosemonophosphate oxidative cycle (19), pentose phosphate (20), and pentose cycle (21), have also been used to describe this pathway.

TABLE 3. $C^{14}O_2$ RATIOS OBTAINED WHEN MAMMARY GLAND SLICES WERE INCUBATED WITH 50 MICROMOLES OF GLUCOSE LABELED IN VARIOUS CARBONS WITH C^{14}

Rat	Hormone treatments			Lactation	$C^{14}O_2$ from G*-1-C^{14} / $C^{14}O_2$ from G-6-C^{14}		$C^{14}O_2$ from G-2-C^{14} / $C^{14}O_2$ from G-6-C^{14}		$C^{14}O_2$ from G-1-C^{14} / $C^{14}O_2$ from G-2-C^{14}	
	Mammotrophin	Δ^1-hydrocortisone acetate	Somatotrophin		Range	Average	Range	Average	Range	Average
	mg.	mg.	mg.							
Normal (7)†	None	None	None	Yes	17.4–20.4	18.3	7.6–11.4	8.8	1.8–2.3	2.1
Hypx (3)	5	0.4	1.0	Yes	9.1–22.0	16.6	8.1–12.6	10.5	1.1–1.8	1.6
Hypx (2)	5	0.4	None	Yes	15.5; 19.0	17.3	8.5; 9.7	9.1	1.7; 1.8	1.8
Hypx (2)	2.5	0.4	1.0	Yes	17.0; 20.0	18.5	9.4; 10.4	9.9	1.6; 2.1	1.9
Hypx (2)	5	None	None	No	1.0; 1.6	1.3	1.1; 1.3	1.2	0.8; 1.2	1.0
Hypx (2)	None	0.4	None	No	1.7; 2.2	2.0	1.2; 1.5	1.4	1.1; 1.9	1.5
Hypx (2)‡	None	0.4	1.0	No		1.8		0.9		2.0
Hypx (1)	None	None	1.0	No		1.3				
Hypx (2)	None	None	None	No	0.8; 1.4	1.1				

* G = glucose.
† Numbers in parentheses indicate the number of rats used.
‡ Slices of the mammary glands of the two rats were pooled for study.

The low glycolytic and lipogenic activity of these nonlactating glands is shown by the data recorded in Table 4. The $C^{14}O_2$ yields and the fatty acid-C^{14} recoveries from the individually labeled hexoses were considerably lower than those observed with the lactating group. The stimulation of lipogenesis from acetate exerted by the addition of glucose to the incubation medium was much less than that observed with lactating rats.

That the metabolism of these enlarged, nonlactating mammary glands differs from that of lactating glands is further brought out by the values for the ratios

$$\frac{C^{14}O_2 \text{ from glucose-1-}C^{14}}{C^{14}O_2 \text{ from glucose-6-}C^{14}} \quad \text{and} \quad \frac{C^{14}O_2 \text{ from glucose-2-}C^{14}}{C^{14}O_2 \text{ from glucose-6-}C^{14}}$$

recorded in Table 3.

The results shown in the last two columns of Table 4 indicate that the hexosemonophosphate oxidative pathway did not participate to any significant extent in the conversion of glucose to fatty acids by slices prepared from the mammary glands of these nonlactating, hypophysectomized rats.

Experiments with Mammary Glands of Hypophysectomized Rats in which Lactation Was Maintained by Daily Postpartum Hormone Injections and then Arrested by Discontinuing Them

In rats 18 and 19 lactation was maintained by hormonal treatments during the first 10 days after parturition. The hormones were withdrawn thereafter, and the rats were killed four days later. At that time, although milk was not expressed from the mammary glands and the young no longer gained weight, the glands had undergone little regression. It should be emphasized that these glands differed but slightly in size from those of the lactating group (Table 1). On the day of necropsy of these rats, no milk was found in the stomachs of their pups. It was assumed that synthesis of milk

TABLE 4. METABOLIC ACTIVITY OF MAMMARY GLAND SLICES PREPARED FROM RATS HYPOPHYSECTOMIZED AT MIDPREGNANCY AND IN WHICH HORMONE TREATMENTS FAILED TO INDUCE LACTATION

(For history of rats and details of hormone injections see Table 1. The incubation procedure is described in Table 2.)

Rat	Hormones injected			Substrates		Per cent of C^{14} recovered as:		Per cent of fatty acid-C^{14} derived from added glucose via Embden-Meyerhof pathway
	Mammotrophin	Δ^1-hydrocortisone acetate	Somatotrophin	Labeled†	Unlabeled	CO_2	Fatty acids	
11	Yes	None	None	Glucose-1-C^{14}	None	0.75	0.10	
				Glucose-2-C^{14}	None	0.94	0.10	
				Glucose-6-C^{14}	None	0.75	0.10	100
				Acetate-2-C^{14}	None	2.27	0.05	
				Acetate-2-C^{14}	Glucose	2.76	0.31	
12	Yes	None	None	Glucose-1-C^{14}	None	2.9	0.40	
				Glucose-2-C^{14}	None	2.0	0.34	
				Glucose-6-C^{14}	None	1.8	0.40	100
				Acetate-2-C^{14}	None	3.2	0.20	
				Acetate-2-C^{14}	Glucose	2.9	0.70	
13	None	Yes	None	Glucose-1-C^{14}	None	4.0	0.08	
				Glucose-2-C^{14}	None	2.1	0.07	
				Glucose-6-C^{14}	None	1.8	0.08	100
				Acetate-2-C^{14}	None	3.6	0.30	
				Acetate-2-C^{14}	Glucose	3.5	0.67	
14	None	Yes	None	Glucose-1-C^{14}	None	2.0	0.08	
				Glucose-2-C^{14}	None	1.8	0.07	
				Glucose-6-C^{14}	None	1.2	0.08	100
				Acetate-2-C^{14}	None	3.6	0.30	
				Acetate-2-C^{14}	Glucose	1.4	0.51	
15+16	None	Yes	Yes	Glucose-1-C^{14}	None	5.5	0.3	
				Glucose-2-C^{14}	None	2.8	0.3	
				Glucose-6-C^{14}	None	3.0	0.3	100
				Acetate-2-C^{14}	None	2.8	0.5	
				Acetate-2-C^{14}	Glucose	2.4	1.3	
17	None	None	Yes	Glucose-1-C^{14}	None	2.0	0.06	
				Glucose-6-C^{14}	None	1.5	0.06	
				Acetate-2-C^{14}	None	3.0	0.03	100
				Acetate-2-C^{14}	Glucose	2.0	0.13	
18	*			Glucose-1-C^{14}	None	2.4	0.1	
				Glucose-6-C^{14}	None	3.0	0.1	
				Acetate-2-C^{14}	None	3.2	0.1	100
				Acetate-2-C^{14}	Glucose	4.2	0.6	
19	*			Glucose-1-C^{14}	None	1.8	0.1	
				Glucose-6-C^{14}	None	1.3	0.1	
				Acetate-2-C^{14}	None	5.8	0.3	100
				Acetate-2-C^{14}	Glucose	5.0	0.4	

* Lactation occurred after parturition in these two rats, as shown in Table 1. Hormone treatments were discontinued for the 4 days before the rats were killed. They did not lactate during those 4 days. Since the glands had not regressed to any considerable extent, they may be regarded as controls for the other nonlactating, hormone-treated rats recorded above.

† From 3.2 to 4.6×10^5 counts per minute of C^{14} per flask.

had ceased, but that some milk had been retained within the duct and alveolar systems.

The metabolic characteristics of the mammary glands of these two rats were the same as those dealt with in the preceding section (Table 4).

The addition of 1 mg. of mammotrophin and 0.5 mg. of Δ^1-hydrocortisone acetate to the incubation medium containing mammary gland slices of rats 18 and 19 did not exert a measurable effect on the conversion of glucose and acetate to CO_2 and fatty acids.

DISCUSSION

In the present investigation we have attempted to evaluate the pattern of glucose and acetate metabolism in the mammary glands of hormonally-

treated, hypophysectomized rats. The following criteria were used for the evaluation: 1) the extent and relative conversions of carbons 1, 2, and 6 of the glucose molecule to CO_2 and fatty acids; 2) the relative participation of the Embden-Meyerhof and hexosemonophosphate oxidative pathways of glycolysis in the conversion of glucose to fatty acids; and 3) the stimulating effect of glucose on lipogenesis from acetate. As judged by these three criteria, the metabolic behavior of the mammary glands of the hormonally-treated, hypophysectomized rats in which lactation was *not* established (those that received mammotrophin alone, or somatotrophin alone, or Δ^1-hydrocortisone acetate) resembled closely the metabolic pattern previously observed by us in the mammary glands of normal, nonlactating rats, i.e., pregnant rats just before parturition and rats in which lactation had just been arrested by weaning of their young (12). These glands had a low capacity for glucose utilization, and they formed fatty acids from glucose exclusively via the Embden-Meyerhof pathway. The *in vitro* addition of glucose induced only a slight stimulation in the conversion of acetate carbon to fatty acids.

The metabolism of the mammary glands of hypophysectomized rats in which lactation was successfully maintained by postpartum hormone treatments was quite similar to that of normal lactating rat mammary glands. Thus, besides establishing a lactational performance amounting to about 50 to 60% of the true milk yield (3), the administration of two hormones, mammotrophin and Δ^1-hydrocortisone acetate, brought about pronounced changes in the mechanism of glucose metabolism. Glucose utilization was extremely high in the glands excised from the lactating, hypophysectomized rats, and the addition of glucose to the medium in which the gland slices were incubated resulted in a pronounced stimulation in lipogenesis from acetate. Half or more of the glucose-derived fatty acids formed in the mammary glands of the lactating, hypophysectomized rats arose via the hexosemonophosphate oxidative pathway. The injection of a third hormone, namely, somatotrophin, seemed to have no additional effect on the metabolic pattern of the lactating gland.

These findings raise the question as to what factor or factors are responsible for the shift in the metabolic pattern of the lactating mammary gland, a shift characterized not only by a greatly augmented capacity for glucose utilization and lipogenesis but also by the induction of an alternative pathway of glycolysis, namely, the hexosemonophosphate oxidative pathway. This shift in metabolic pattern does not appear to depend upon growth and development *per se* of the mammary gland nor upon the action of any one hormone. According to Cowie and Folley (22), lactation can be separated into two phases: one in which milk is synthesized and secreted into the alveolar lumen and then moved along the ducts; and a second in which milk is removed by the young or by other means. Probably the only additional hormone influencing this latter phase is oxytocin. Our rats had

regenerated this mechanism (hypothalamic secretion) to varying but effective degrees. Since the lactating as well as the nonlactating rats were supplied throughout the postpartum period with six new-born rats, it seems likely that the stimuli or factors responsible for giving rise to the metabolic pattern of the lactating mammary gland are connected with the first process of lactation, namely, milk formation and secretion.

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SUPPRESSION OF STEROID DIABETES BY WALKER CARCINOMA IN THE RAT

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ABSTRACT

Male Sprague-Dawley rats were tube-fed a medium carbohydrate diet twice daily. Steroid diabetes was induced by subcutaneous injection of cortisone acetate each morning. Implantation of Walker Carcinoma 256 into each groin was followed by marked suppression of glycosuria after the tumor began to grow. The growth of tumor was suppressed in proportion to the dose of cortisone. At the end of 10 days the average weight of tumor in rats without cortisone was 25.2 gm.; in similar rats given 15 mg. of cortisone daily the average weight of tumor was 3.31 gm., and the average amount of glucose excreted on the 10th day was 0.61 gm. In rats without tumor given 15 mg. of cortisone daily the average amount of glucose excreted on the 10th day was 5.68 gm. In some tumor hosts the glycosuria disappeared as the tumor grew slowly. Cortisone acetate (15 mg. daily) was tested in animals with established tumors. After tumor growth for 12 days and longer glycosuria could no longer be induced by the steroid. When rats with steroid diabetes were stressed by the excision of skin there was a moderate suppression of glycosuria.

THIS study grew out of speculations about the comparative effects of steroid diabetes, pancreatic diabetes, and alloxan diabetes upon the growth of the Walker carcinoma in the rat. The pancreatic and alloxan forms, characterized by insulin deficiency, are known to suppress the growth of tumor in proportion to the severity of the diabetes. (1) As these tumors grow, even at a reduced rate, the glycosuria becomes less severe. A dramatic suppression of tumor growth is achieved by treatment with large doses of cortisone from the time the tumor is implanted. In the experiments reported here, the glycosuric response to cortisone was markedly suppressed by the growing tumor. When rats with steroid diabetes were stressed by the excision of skin, there was a moderate suppression of glycosuria.

METHODS

Male rats (300–320 gm.) of the Sprague-Dawley strain were placed in individual metabolism cages and adapted to the tube-feeding of a medium carbohydrate diet (2) in standard amounts (13 cc.) twice daily. Steroid diabetes was induced by the subcutaneous injection of cortisone acetate (Upjohn) in aqueous suspension following each morning feeding. Infection, which may develop in rats with severe hypercorticalism, was prevented by daily doses of penicillin (5000 u) and streptomycin (5 mg.). Twenty-

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TABLE 1. EFFECT OF WALKER CARCINOMA 256 ON STEROID DIABETES FOR 10 DAYS. (AVERAGES AND STANDARD ERRORS)

Group	Condition	N rats	Mg./day cortisone	Body-wt., gm.		Urinary glucose: Mg. per day										Mg. spleen	Gm. tumor
				Initial	Final	1	2	3	4	5	6	7	8	9	10		
1	Tumor only	8	0	284 ±3.89	328 ±5.71	0	0	0	0	0	0	0	0	0	0	1940 ±124	25.8 ±2.84
	No tumor	6	5	285 ±4.14	265 ±7.0	0	28 ±11	373 ±220	827 ±397	1435 ±461	2037 ±629	2125 ±538	2012 ±471	1980 ±494	1785 ±302	397 ±27	
	Tumor	6	5	288 ±4.45	282 ±7.9	0	0	570 ±276	1110 ±500	1370 ±507	1285 ±482	992 ±351	817 ±301	612 ±227	218 ±37	1082 ±189	11.14 ±0.91
2	No tumor	6	7.5	286 ±2.9	251 ±8.2	0	0	560 ±321	998 ±648	1460 ±794	2722 ±1077	3400 ±1094	3062 ±1069	3828 ±1360	3842 ±1264	353 ±31	
	Tumor	6	7.5	286 ±2.9	280 ±10.0	0	0	675 ±383	1148 ±714	2359 ±1004	2393 ±955	1525 ±685	720 ±350	428 ±91	172 ±12	1407 ±228	12.79 ±2.54
3	No tumor	7	10	287 ±7.4	243 ±10.6	0	70 ±69	927 ±343	1807 ±607	3409 ±766	4387 ±770	4390 ±680	4584 ±692	4539 ±705	4481 ±626	311 ±32	
	Tumor	7	10	286 ±4.2	247 ±2.7	89 ±48	24 ±24	1297 ±402	2801 ±740	2801 ±728	3687 ±687	2819 ±662	1781 ±388	769 ±288	497 ±267	852 ±24	9.39 ±1.34
4	No tumor	6	15	284 ±2.4	227 ±4.4	0	0	780 ±341	3485 ±569	5345 ±318	6825 ±310	7053 ±256	6240 ±364	6123 ±333	5525 ±359	222 ±8.57	
	Tumor	5	15	287 ±2.0	236 ±4.9	0	0	1756 ±529	3844 ±655	5574 ±709	5407 ±801	4377 ±641	2599 ±887	1349 ±137	1037 ±66	612 ±49	3.93 ±0.68

TABLE 2. EFFECTS OF WALKER CARCINOMA 256 ON STEROID DIABETES: INTACT MALE RATS GIVEN 15 MG. CORTISONE ACETATE DAILY BEGINNING AT DIFFERENT PERIODS AFTER IMPLANTATION OF TUMOR IN EACH GROIN. RESULTS EXPRESSED AS NUMBER OF DAYS EACH INDIVIDUAL RATS EXCRETED GLUCOSE

Days delay in starting cortisone							
7	8	9	10	11	12	13	14
5	5	8	4	4	0	0	0
4	4	8	3	1	0	0	0
4	4	4	2	1	0	0	0
3	2	0	0	1	0	0	0
2	1	0	0	0	0	0	0
2	1	0	0	0	0	0	0
1	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0

four-hour samples of urine were collected each morning and the amount of urinary glucose was determined by the method of Benedict (3). Walker Tumor 256 was injected as a sterile undiluted homogenate into the upper thigh of each hind leg. At the end of each experiment the tumor was dissected free from the muscle and weighed. Some organs were weighed at necropsy. Excision of skin was done under ether anesthesia by clean techniques. The wounds were closed by continuous silk sutures.

EXPERIMENTS AND RESULTS

In experiment 1 (Table 1) cortisone acetate was given in doses of 5, 7.5, 10 and 15 mg. per rat per day for 10 days in order to establish the relationship of tumor growth to the severity of the glycosuria. Comparisons of rats with and without tumors were made at the same time. Tumors were implanted on the day treatment with cortisone was started. One group of tumor hosts did not receive cortisone.

Cortisone suppressed body-weight to an extent that correlated with dosage. Tumor hosts did not lose as much weight on cortisone treatment as did rats without tumors. Cortisone alone caused marked atrophy of the spleen. In contrast, the spleens of the tumor hosts enlarged. When tumor hosts received cortisone, the average spleen weight was intermediate between those produced by either condition alone.

Cortisone caused suppression of tumor growth in proportion to the amount injected. This was very striking at the 15 mg. per day level. These small tumors appeared to be completely necrotic by gross examination. However viable tissue remained, for in related experiments it was found that when cortisone treatment was stopped, each neoplasm began to grow rapidly.

Glycosuria began on the 2nd and 3rd day in all rats receiving these high doses of cortisone. Higher values were obtained by the higher doses. In rats without tumors, fairly steady values were maintained from the 6th to the 10th days. Initially, the tumor had no observable effect upon the glycosuria. With great regularity, however, on about the 6th day the urinary glucose of the tumor hosts began to fall. This coincided with the time the tumor became visible and grew rapidly. By the 10th day the glycosuria had either disappeared or was mild in all tumor hosts.

Experiment 2 (Table 2) was designed to find a critical period of tumor growth after which cortisone treatment would no longer elicit glycosuria. These rats were each given 15 mg. of cortisone acetate beginning at varying times after tumor implantation. The tumors attained a size which was proportional to the time they had been growing. Cortisone given on or before the 7th day of tumor growth induced glycosuria for at least one day in all rats. Thereafter progressively fewer rats showed this minimal response, and all rats which had tumors for 12 days and longer failed to develop glycosuria. A second series of rats which were hosts to tumors for 12 days and longer were given as much as 25 (10 rats) and 40 mg. (5 rats) of cortisone per day, doses which would kill most normal rats, but it was well tolerated

and produced no glycosuria except for about 300 mg. of glucose on one day by a rat given 40 mg. of cortisone acetate daily. Final tumor weights are not given, but it was clearly apparent that when the tumor was well established prior to beginning treatment with cortisone, it neither regressed during treatment nor was its subsequent growth strikingly inhibited as occurred in rats treated from the time the tumor was implanted.

Experiment 3 was done to test the effect of a stressor other than a tumor on steroid diabetes. It was thought that the marked effect of the neoplasm upon steroid diabetes could be in part non-specific, and might be simulated by other severe stressors. In a preliminary experiment 13 rats were each given 15 mg. of cortisone acetate per day for 11 days. On the 6th day, 10 of the rats were stressed by the excision of skin from the nape of the neck

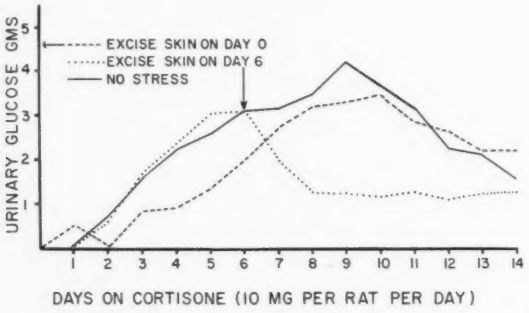

FIG. 1. Effect of stress on the glycosuria of rats with steroid diabetes.

to the base of the tail. In 3 of these rats the skin was slit open but none removed; in 4 rats, approximately 5 gm. of skin were removed from each before closing the wound; and in 3 rats, approximately 10 gm. of skin were excised before closing the wound. Three rats served as controls. In the unoperated rats there was, as previously reported (3), some spontaneous decrease from the peak of glycosuria that occurred about the 6th or 7th day in rats with steroid diabetes. All rats stressed by excision of skin showed a fall in glycosuria greater than the spontaneous fall observed in the control group.

In a second part of this experiment (Fig. 1), 25 rats were each given 10 mg. of cortisone acetate daily for 14 days. Six rats were unoperated controls; 10 rats were each subjected to the excision of approximately 14 gm. of skin on the day the injection of steroids was started; and 9 rats were each subjected to the excision of approximately 10 gm. of skin at the end of the 7th day of steroid injection. The area of skin removed was about the same in each group; the smaller weight of skin removed in the 2nd group of operated rats was due to thinning of skin caused by hypercorticalism. The unstressed rats treated with cortisone did not excrete glucose during the

first 48 hours. The rats stressed on the first day of cortisone treatment each excreted small amounts of glucose during the first 24 hours. This temporary glycosuria probably represents glycogenolysis in a liver loaded with glycogen. Subsequent observations have related this first-day reaction to the severity of the stress, and it can occur in the absence of cortisone treatment. Despite this initial glycosuria, the daily values for urinary glucose remained lower in the stressed animals than in the controls until, as the wound healed, the average value approached that of the controls. When the stress was delayed until the level of glycosuria was high—on the 6th day—the glycosuria showed a marked drop, and low levels were maintained throughout the remainder of the experiment.

DISCUSSION

Each of the two kinds of stress tested had a suppressive effect on at least one of the biologic effects of cortisone overdosage, namely, glycosuria. Can the suppression of glycosuria by the growing tumor be due to increased oxidation of glucose by the tumor itself? This may be a factor, but it seems implausible that an almost completely necrotic tumor of 3 or 4 gm. weight could oxidize 5 gm. or more of glucose daily, such amounts being excreted by the non-tumor controls. Similarly, it is implausible that the protein content of these small tumors is sufficient to explain the shift in glycosuria as due to less protein for glyconeogenesis. More probably the toxic effects of the tumor cause changes in metabolic activity throughout the organism, or there may be a more direct effect such as inactivation of cortisone itself.

The observation that excision of skin also suppresses glycosuria may mean that the effect of the tumor is that of a non-specific stress. The effect of the tumor is more striking, but it is a more severe stressor than excision of skin. Although tumor growth abolished the glycosuric response to very large doses of cortisone, the milder effects of surgical stress were more striking in rats given 10 mg. of cortisone daily than in rats given larger doses. The effect of stress upon steroid diabetes is best shown when the dose of steroid causes a steady but not maximal glycosuria in control animals. Although the quantitative relationship between cortisone dosage and the degree of stress required to suppress steroid diabetes was not established in these experiments, it seems probable that for a given stressor a level of cortisone dosage can be defined for which the suppression of glycosuria in response to the stressor is maximal.

The adrenal cortices of the tumor host become greatly hypertrophied as the tumor grows. It might be supposed that the secretory activity of the adrenal cortices is increased and that signs of hypercorticalism are more likely to occur. On the contrary, exposure of rats overdosed with cortisone to either tumors or to surgical stress caused suppression of one sign of hypercorticalism—glycosuria.

The author interprets these results as fitting a hypothesis which has

evolved out of previous work in this laboratory (4); the adrenal cortical response to stress serves to meet the increased need of the organism for glucocorticoids and does not result in hypercorticalism. Did the stress used in these experiments make the animals overdosed with cortisone more eucorticoid? It may be possible to simulate these results with other nonspecific stressors. Can other symptoms of hypercorticalism be suppressed by severe stress? It is recognized that glycosuria is a secondary expression of hypercorticalism. Adequate testing of the hypothesis under study will require further research with other criteria of hypercorticalism and other stressors.

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CORTICOSTEROID PRODUCTION BY INTERRENAL TISSUE OF TELEOST FISHES[1]

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ABSTRACT

Evidence has been obtained suggesting that the interrenal gland of teleost fishes is capable of secreting adrenocortical steroids. Several species of teleosts were used, and the anterior or head kidneys, containing the interrenal tissue, were incubated *in vitro*. Tentative identification of steroid secretory products was achieved, in most cases by chromatography of extracts in toluene-75% methanol and development with triphenyltetrazolium chloride.

A single spot, having the same mobility as cortisol, was obtained from the incubated tissue of *Anoplopoma fimbria* (sablefish). Spots corresponding to cortisol and cortisone were observed after incubation of the head kidneys of *Mugil cephalus* (mullet). Cortisone was tentatively identified in extracts of the incubated tissue from *Tilapia mossambica* (cichlid), as well as a substance with a polarity similar to, but not identical with, corticosterone. A fourth species, *Bodianus bilunulatus* (a'awa), failed to yield any detectable steroid, despite the relatively large number of animals used.

ALTHOUGH the interrenal organ of teleosts is thought to represent adrenocortical tissue (1), there has been no direct evidence that this gland does in fact secrete adrenocortical hormones. In 1954 Hatey (2) and Fontaine and Hatey (3) demonstrated the presence of 17-hydroxycorticosteroids in the blood of salmon (*Salmo salar*), and since that time further information has been obtained concerning the presence of adrenal steroids in teleost blood. Cortisol has been found in the blood of several species: *Gadus morrhua* (4), *Pseudopleuronectes americanus* (5), *Cyprinus carpio* (5, 6, 7), and *Fundulus heteroclitus* (7). Hatey (6) found traces of corticosterone as well as cortisol in the blood of *Cyprinus carpio*. Both cortisol and cortisone have been demonstrated in the blood of *Oncorhynchus nerka* (8), and of *O. tschawytscha* and *Salmo gairdnerii* (9). Although changes in interrenal histology have been correlated with alterations in the level of circulating adrenal steroids (3, 9), these observations offer no direct proof that the interrenal is the source of these hormones.

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[4] Contribution No. 123 from the Hawaii Marine Laboratory.

Recently, Phillips and Mulrow (10) showed that anterior kidney tissue of the killifish *Fundulus heteroclitus* is capable of converting progesterone to adrenal steroids (cortisol, cortisone, and aldosterone) *in vitro*. This observation is the first direct evidence that enzymes characteristic of adrenocortical tissue are present in the region of the kidney containing interrenal gland.

In the present study, we have attempted to obtain evidence that the interrenal is capable of elaborating adrenocortical steroids, and to compare the secretory patterns in several teleosts, by adaptation of the Hofmann incubation method (11) to fish tissues. Four teleost species were used: *Anoplopoma fimbria* (sablefish: marine), *Bodianus bilunulatus* (a'awa or spot wrasse: marine), *Mugil cephalus* (mullet: euryhaline littoral) and *Tilapia mossambica* (cichlid: freshwater).

MATERIALS AND METHODS

Animals

Anoplopoma fimbria, the sablefish, of the family Anoplopomidae, were collected west of San Juan Island in August of 1958 and of 1959[5] at the University of Washington's Friday Harbor Laboratories. *Tilapia mossambica*, a cichlid introduced into Hawaii, were obtained from a reservoir near Ewa, Oahu, Hawaii, through the cooperation of Mr. Kenji Ego and Mr. Richard Yoshida of the Territorial Division of Fish and Game at the end of 1958 and the beginning of 1959. Some of these freshwater fish were moved into seawater aquaria 6 weeks prior to sacrifice. *Mugil cephalus*, the mullet, of the family Mugilidae, were obtained in late 1958 and early 1959 by commercial seining from the shores of Kaneohe Bay on the windward side of Oahu, Hawaii, with the help of Mr. Lester Zukeran of the Hawaii Marine Laboratory. *Bodianus bilunulatus*, the a'awa or spot wrasse, of the family Labridae, were trapped by commercial fishermen off Oahu, Hawaii, in the spring of 1959, and were made available by the cooperation of Mr. Spencer Tinker and his staff of the Honolulu Aquarium.

Incubation

In all species except *Anoplopoma fimbria*, the osmotic pressure of the blood was determined by a modification of the melting point method of Gross (12). A concentrated stock solution of medium was made, containing the following concentrations of solutes (mM./l.): NaCl, 200.0; KCl, 5.0; $CaCl_2$, 3.0; $MgCl_2$, 2.0; $NaHCO_3$, 1.0; NaH_2PO_4, 10.0; glucose, 6.0. This solution was made approximately isosmotic to the blood by dilution with distilled water. The final concentrations were as follows: *Tilapia* from fresh water, 0.16M; *Tilapia* from sea water, 0.21M; *Mugil* (marine, littoral), 0.22M; *Bodianus* (marine), 0.22M. In one experiment with fresh-water *Tilapia*, the medium was 0.20M. No blood determinations were made on *Anoplopoma* (marine), and a 0.11M solution was used.

Tilapia, *Mugil*, and *Bodianus* all possess a pair of discrete head kidneys, which were dissected out and cut into small pieces in the incubating medium. Usually the head kidneys of two animals were placed in a single flask (10 ml. Erlenmeyer) containing 3 ml. of medium. The flasks were gassed with pure oxygen for 5 minutes, stoppered, and incubated in a Dubnoff metabolic incubator at room temperature. The medium was changed

[5] We are indebted to Professor Paul Illg and Mr. Hugh Stanky for the collection of additional sablefish material.

and reoxygenated at the end of ½ hour, and again after the next 1½ hours of incubation. After a final 1½ hours, the tissue and the three changes of medium were pooled and frozen. In some experiments, the medium from the first ½ hour of incubation was discarded.

Inasmuch as there is no distinct head kidney in *Anoplopoma*, the expanded anterior portion of the trunk kidney was removed. The tissue from each animal was placed in a separate flask (25 ml.) with 6 ml. of medium. The tissues were incubated for 2½ hours at room temperature by bubbling oxygen directly into the medium through a hypodermic needle. The medium was not changed during the incubation. Tissues and media were stored frozen prior to extraction.

Extraction

The medium and tissue from each flask was extracted three times with two volumes of ethyl acetate. The ethyl acetate extract was washed with 3 ml. of 0.05N Na_2CO_3, and then washed twice with 3 ml. of distilled water. The extracts from several incubation flasks were pooled, and the ethyl acetate distilled off under vacuum at 40–45° C. The residue was further purified by partitioning between distilled water and n-hexane (Skellysolve B). The aqueous phase was re-extracted three times with ethyl acetate. The final extract was again dried down, and stored in the deep-freeze.

Chromatography

The residue was dissolved in 50 μl. absolute methanol and spotted on Whatman #20 filter paper. Standard steroids were applied to lanes running parallel to the unknown samples. Most of the chromatograms were run for 6–8 hours at room temperature in a toluene-75% methanol system (13), after equilibration overnight. One of the pooled *Tilapia* extracts that had been stored in the deep-freeze for 2½ months was run in cyclohexane-benzene-methanol-water (100:50:100:25) (10) for 24 hours at room temperature. The *Bodianus* extract was first chromatographed in cyclohexane-benzene-methanol-water for 24 hours; following this, three areas (corresponding to the location of cortisol plus cortisone, corticosterone, and 11-dehydrocorticosterone) were eluted with methanol and separately rechromatographed in toluene-75% methanol.

In most cases, identification of steroids was achieved by comparing their relative mobilities with those of the standards run simultaneously after development with triphenyltetrazolium chloride (TPTZ) (14). The *Tilapia* strip run in cyclohexane-benzene-methanol-water was sprayed with 2N NaOH, and the fluorescent spots observed under ultraviolet light (15). The *Bodianus* chromatograms were cut lengthwise in half; one half was treated with TPTZ, the other examined by the soda-fluorescence method.

Histology

Representative paraffin sections were made of the head kidneys or anterior trunk kidneys, corresponding to the regions taken for incubation. The presence of interrenal gland was confirmed, and the nature of the surrounding tissues, also included in the incubation, was determined. Tissues were fixed in Bouin's and stained with alum-hematoxylin and eosin.

RESULTS

Anoplopoma fimbria. The anterior kidneys of 17 animals were incubated, and two chromatograms were run in the toluene-75% methanol system (Fig. 1). A single spot, with a mobility identical to that of cortisol in this system, appeared on each chromatogram after development with TPTZ.

The histologic appearance of the interrenal gland and surrounding tissues

FIG. 1. Chromatographic analysis of steroids from *Anoplopoma fimbria*. Stippled spots represent the unknown substances, black areas correspond to the standard steroids run simultaneously. N indicates the number of animals used in each experiment. The incubation medium was 0.11M. Chromatograms were run in toluene-75% methanol and developed with TPTZ.

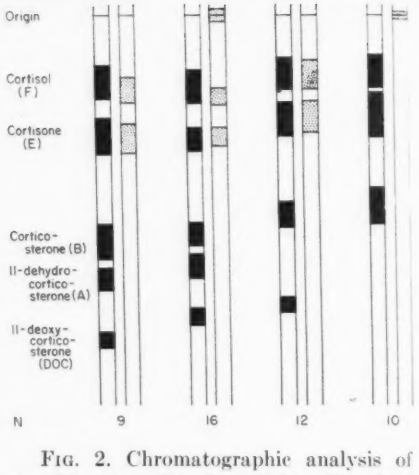


FIG. 2. Chromatographic analysis of steroids from *Mugil cephalus*. Standard and unknown steroids are shown as in Figure 1. The incubation medium was 0.22M. All chromatograms were run in toluene-75% methanol and developed with TPTZ.

is shown in Figure 4. Strands of interrenal cells are found surrounding small veins, scattered through the anterior kidney. A few chromaffin cells may be found in these areas, but most of the chromaffin tissue is embedded in the walls of larger blood vessels. Kidney tubules and hemopoietic tissue, predominantly lymphoid, make up most of the surrounding tissue of the anterior kidney.

Mugil cephalus. 47 animals were used, and 4 chromatograms were run in toluene-75% methanol (Fig. 2). On three of these, TPTZ-positive spots were found opposite the cortisol and the cortisone standards. Two of the chromatograms also had TPTZ-positive material remaining at the origin. The fourth chromatogram revealed only the spot on the starting line. The significance of this spot remains to be determined, as all our standard steroids moved off the origin in this chromatographic system.

Figure 5 shows the histology of the mullet head kidney, which is morphologically distinct in this species. Large masses of interrenal tissue surround the major branches of the postcardinal veins. Small clumps of lightly stained cells, presumably chromaffin, are found close to the blood vessel lumina. No renal elements are found in this region. The surrounding hemopoietic tissue is chiefly myeloid, but lymphoid tissue is also prominent.

Bodianus bilunulatus. Although the extracts from 34 animals were pooled

ınd chromatographed together, no steroids were detected by either TPTZ ɔr the soda-fluorescence methods.

The histology of the head kidney is shown in Figure 7. Although the ıead kidney is morphologically discrete, it is predominantly a mass of renal ubules, with lymphoid tissue visible between these. Clumps of pale chromaffin cells are widely scattered through the interrenal, which surrounds ıany of the larger veins and their branches.

Tilapia mossambica. 129 animals were used, and 7 chromatograms run (Fig. 3). After chromatography in cyclohexane-benzene-methanol-water ınd development by the soda-fluorescence method, two spots were ob-

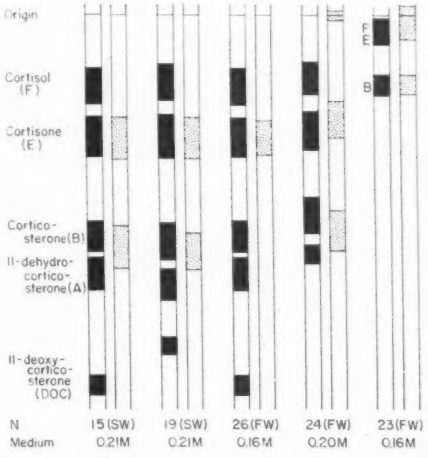
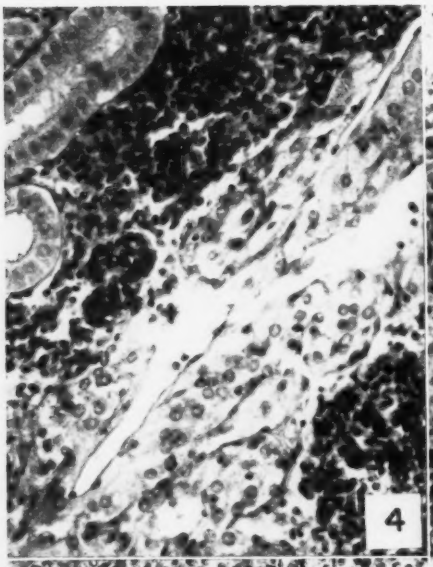


FIG. 3. Chromatographic analysis of steroids from *Tilapia mossambica*. Standard and unknown steroids are shown as in Figure 1. N indicates the number of animals used, SW refers to animals maintained in sea water for 6 weeks prior to incubation of the head kidneys, FW refers to fish taken directly from fresh water. The first four chromatograms were run in toluene-75% methanol and developed with TPTZ. The last chromatogram was run in cyclohexane-benzene-80% methanol and developed by the soda-fluorescence method.

served. The less polar of these was found opposite the corticosterone standard, and the polar component appeared as a band from the origin through the region occupied by cortisol plus cortisone. Two of the toluene-75% methanol chromatograms, representing 8 and 14 animals, revealed no spots after treatment with TPTZ, and are not shown in Figure 3. A spot corresponding to cortisone was seen on a third, and a fourth toluene-75% methanol chromatogram showed 3 spots: one at the origin, one corresponding to cortisone, and one slightly less polar than corticosterone. All of these were TPTZ-positive.

Both experiments run with seawater-adapted *Tilapia* revealed a TPTZ-positive spot corresponding to cortisone, and one having a slightly greater mobility than corticosterone.

Figure 6 shows the histology of the interrenal and the head kidney, which is discrete in this species. The hemopoietic tissue appears to be precominantly myeloid, and no renal elements are present in the tissue associated with the interrenal. The interrenal resembles that of *Mugil cephalus*, except that no chromaffin cells have been distinguished in this area.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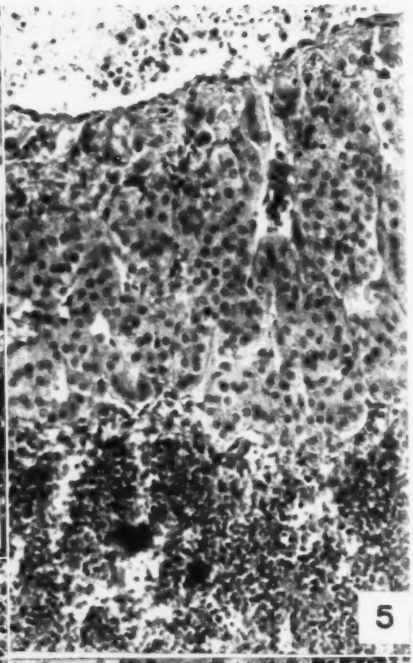
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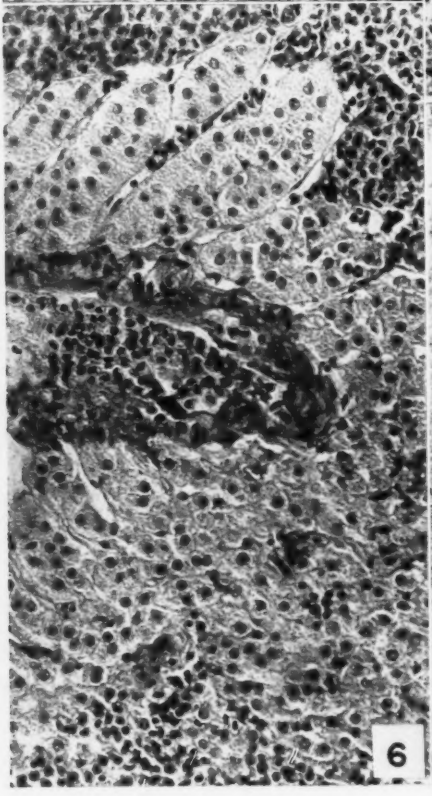
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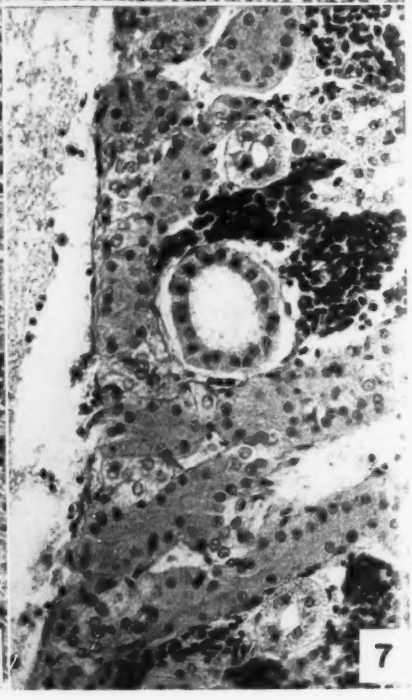
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DISCUSSION

The results (summarized in Table 1) presented herein strongly suggest that the interrenal gland of teleosts is capable of producing corticosteroids. This is further substantiated by the fact that in the rainbow trout (*Salmo gairdneri*), relatively large quantities of control tissue (trunk kidney or liver) fail to yield detectable amounts of steroid (16). It should be emphasized, however, that the identification of the secretory products is still

TABLE 1. CORTICOSTEROID SECRETORY PATTERN AND HISTOLOGIC COMPOSITION OF ANTERIOR KIDNEYS IN SEVERAL TELEOST FISHES

Species	Normal habitat	Total no.	No. of chromatograms	Steroids indicated				Presence of discrete head kidney	Histology		
				Cortisol	Cortisone	Corticosterone	11-dehydrocorticosterone		Presence of kidney tubules	Predominant hemopoietic tissue	
										Lymphoid	Myeloid
Mugil cephalus	marine	47	4	+	+	−	−	+	−	+	++
Anoplopoma fimbria	marine	17	2	+	−	−	−	−	+	+	
Bodianus bilunulatus	marine	34	1	−	−	−	−	+	+	+	
Tilapia mossambica	freshwater	107[1]	5[1]	−	+		?	+	−		+
Salmo gairdneri[2]	freshwater	80	7	+	±	±	−	−	−	+	+

[1] Two additional chromatograms, using 22 more fish, revealed no spots.
[2] Nandi, J. Unpublished observations.

tentative, in most cases based only upon the mobility of the substances in a single solvent system, and upon their reaction with TPTZ. In all cases only small quantities of material, in the order of 2–5 micrograms (based on comparison with known quantities of standard), were obtained.

Despite the preliminary nature of these results, they are in general agreement with the observations of other workers on the corticosteroids present in teleost blood. The predominance of relatively polar steroids (cortisol and cortisone) is evident in all teleosts thus far examined. However, *Tilapia* represents the first teleost in which no evidence of cortisol secretion has

FIG. 4. Histology of the anterior kidney of *Anoplopoma fimbria*, showing interrenal cells. Kidney tubules are present at the upper left. The remainder of the tissue is chiefly lymphoid. H. and E. ×370.

FIG. 5. Histology of the head kidney of *Mugil cephalus*. The lumen of a vein appears at the top. The interrenal cells extend as cords into the surrounding tissue which contains both myeloid and lymphoid elements. H. and E. ×370.

FIG. 6. Histology of the head kidney of *Tilapia mossambica*. Cords of interrenal cells surround a vein, and extend into the surrounding tissue which is predominantly myeloid. H. and E. ×370.

FIG. 7. Histology of the head kidney of *Bodianus bilunulatus*. The lumen of a vein is seen at the left. Masses of interrenal cells lie along this vein and also its branches, as seen at the lower right. The pale cells visible in this tissue are presumably chromaffin. Several kidney tubules and a collecting duct can be seen. The hemopoietic tissue is chiefly lymphoid. H. and E. ×370.

been found. Since only a few species have as yet been studied, no correlation can be made between the observed secretory pattern and the habitat, physiology, or taxonomic position of the species. That the secretory pattern of a given species does not alter with a change in habitat is suggested by the fact that the major components were the same in both freshwater and seawater-adapted *Tilapia*. There is some indication, however, that the secretory capacity of the tissue may have been somewhat greater in the seawater-adapted fish. Hane and Robertson (9) also found no qualitative variation in the blood corticoids of salmon under different environmental conditions.

A number of factors complicates the use of *in vitro* methods with teleost material. The histology of the incubated tissue reveals the presence of several tissue types other than interrenal: chromaffin tissue, hemopoietic tissue, and in some cases renal tubules are present. Work currently in progress on the rainbow trout (*Salmo gairdnerii*) indicates that the presence of these tissues may result in considerable loss of extractable steroid through some type of tissue binding. The possibility of subsequent metabolic alterations of the secreted steroids by the surrounding tissues, particularly by kidney tubules, also must be considered. These factors undoubtedly account in part for the low yield of steroids in our system. Extensive conjugation of steroids by the masses of renal tubules of the *Bodianus* head kidney, for example, may have been responsible for our failure to obtain sufficient quantities for identification in this species.

The microanatomic nature of the anterior kidney cannot be predicted by gross anatomic considerations. The discrete head kidneys of *Tilapia* and *Mugil* are free from renal elements, but the equally discrete head kidney of *Bodianus* is largely renal in structure. On the other hand, the anterior end of the trunk kidney in *Salmo* shows no renal component, whereas that of *Anoplopoma* is definitely renal. Variations in the nature of the hemopoietic "background" tissue are also considerable (see Table 1).

As in any *in vitro* system, one cannot assume that the secretory products observed correspond to those normally secreted by the interrenal gland. Preliminary experiments with the rainbow trout, using the same methods described herein, indicate the presence of cortisol, and possibly of cortisone and of small amounts of corticosterone (16). This compares favorably with the finding of cortisol and cortisone in the blood of the same species by Hane and Robertson (9).

Because of the small yield of steroid, our results probably represent only the major products of secretion from the teleost interrenal. This is equally true of the analyses of fish systemic blood. Thus, we have not yet been able to determine, for example, whether aldosterone is secreted by the teleost interrenal, although the finding by Phillips and Mulrow (10) that progesterone can be converted to aldosterone by anterior kidney tissue is highly suggestive. In addition, Phillips, Holmes and Bondy (17) have evi

dently detected aldosterone in the blood of male sockeye salmon. The yield of steroids from our incubations might well be increased with modifications of the incubating medium by addition of corticotropin preparations, by addition of substrates involved in steroid biosynthesis, and by alterations in ionic composition. Mammalian corticotropin preparations were not used in the present study to avoid possible pharmacologic effects, but may provide information of considerable interest (18). The addition of small amounts (approximately 0.1 Armour ACTHAR unit per 100 mg. of anterior kidney tissue) of ovine corticotropin to the incubation medium apparently increased, but did not qualitatively alter, the secretory products of the trout interrenal (16).

Further experiments are now in progress to provide more complete identification of the teleost corticoids, as well as to further define the interactions of non-interrenal tissue with steroids in our *in vitro* system.

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NOTES AND COMMENTS

EFFECT OF REMOVAL OF BURSA FABRICII ON DEPLETION OF ADRENAL ASCORBIC ACID IN ACTH-TREATED CHICKS

ABSTRACT

The bursa of Fabricius was surgically removed in a number of chicks; bursectomized, and bursae-intact chicks were equally treated with ACTH injections and their adrenal ascorbic acid subsequently examined. A highly significant depletion of the adrenal ascorbic acid was obtained in the bursectomized birds; no response was noted in the chicks with intact bursae. It was concluded that the bursa of Fabricius plays an inhibiting role in the elicitation of adrenal ascorbic acid depletion reaction to ACTH treatment in young birds.

Several workers have reported that birds differ from other animals in their reaction to ACTH or stress, since they do not deplete their adrenal ascorbic acid (1–5). On this basis the study of stress in birds has been difficult because of the lack of suitable criteria for the evaluation of stress in fowl.

Recently it was demonstrated that adrenal ascorbic acid depletion could be accomplished in ACTH treated laying hens, but not in young chickens (6). The cause of failure of young birds to respond in this respect remained an unsolved problem.

Since the function of the bursa of Fabricius, a structure peculiar to birds, existing up to 5–6 months, is not understood, the authors examined the correlation of the bursa with the lack of adrenal ascorbic acid depletion in ACTH treated young chickens.

MATERIALS AND METHODS

Thirty-six male White Leghorn chicks, 6 weeks old, were used. Eighteen of these birds were bursectomized at the age of 2 weeks, and kept for 4 weeks during recovery. The bursectomy was performed by the method of Chang, Rheins and Winter (7). The day after the removal of the bursa the chicks ate and drank well and their later development was of the same rate as the controls. All chicks were kept in batteries and fed a commercial starter mash.

The bursectomized chicks were divided into 3 groups, each consisting of 6 birds. Group I served as controls for groups II and III; each chick of this group received 1 ml. of saline solution intravenously and was killed one hour later. Those from group II were injected intravenously with 1 ml. of 10 I.U. of fast acting corticotropin (Organon, Holland) one hour before sacrifice, while the birds of group III were treated with "prolonged action" corticotropin as follows: during 24 hours, each chick was injected intramuscularly with 3 equal doses of 10 I.U. of corticotropin suspended in 5% beeswax in peanut oil which was warmed and stirred before injection. The birds were killed 24 hours after the last injection.

Groups IV, V and VI, each consisting of 6 chicks with intact bursae were treated in the same manner and received the same doses as Groups I, II and III respectively.

All birds were killed by cervical dislocation. Both adrenals of each bird were removed, weighed together and homogenized. No individual body-adrenal weight relations were performed as all birds were approximately of the same weight. The adrenal ascorbic acid determinations were carried out by the method of Roe and Kuether (8), using Norit as oxidizing agent. Readings were taken on a Hilger electrophotometer.

RESULTS AND COMMENTS

The data in Table 1 show that when compared to the saline-treated bursectomized chicks both the fact acting and the prolonged-acting ACTH treatment caused a 49.5% and 41.37% depletion in total adrenal ascorbic acid (Groups II and III); the concentration decreased 57.3% and 45.8% respectively. Statistically, both results are highly significant. No significant changes occurred in the adrenal ascorbic acid of chicks with intact bursae in Groups IV, V and VI treated in the same manner.

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TABLE 1. ADRENAL ASCORBIC DEPLETION IN ACTH- TREATED INTACT AND BURSECTOMIZED CHICKENS

Groups and treatment	No. of chickens	Mean* Weight		Adrenal Ascorbic acid			
		Mg.	% diff.	Content, μg.	% diff.	Concentration, mg. %	% diff.
I. Bursectomized, saline control	6	59±3	—	84.6±8	—	143.3	—
II. Bursectomized, ACTH fast acting 10 IU.	6	70±14	18.6	42.7±3	49.5	61.0	57.3
III. Bursectomized, ACTH prolonged action 3/10 IU.	6	63.7±2	7.96	49.6±4	41.37	77.7	45.8
IV. With intact bursae saline control	6	60.0±6	—	91.16±7	—	151.9	—
V. With intact bursae ACTH fast acting 10 IU.	6	57.3±3	4.16	88.34±7	3.09	144.1	1.4
VI. With intact bursae ACTH prolonged actions 3/10 IU.	6	62.0±5	3.33	81.45±5	10.65	131.3	13.5

* ± Standard Errors.

The function of the bursa of Fabricius in birds is obscure. Different workers ascribe to it endocrine functions related to growth and sexual development (9, 10), lymph gland and thymus-like functions (11, 12, 13), and an involvement in antibody production (14).

The results reported demonstrate clearly that the bursa of Fabricius prevents the adrenal ascorbic acid depletion in young chickens caused by ACTH. Whether the bursa plays a major role in stress, is a challenging question.

The authors suggest that adrenal ascorbic acid depletion in bursectomized chicks be used for evaluating the magnitude of acute and chronic stressors in birds.

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EFFECT OF GRADED DOSES OF ANTERIOR PITUITARY EXTRACT ON THYROID WEIGHT AND THYROID/SERUM IODIDE RATIO IN THE HYPOPHYSECTOMIZED RAT[1]

ABSTRACT

Graded doses of crude extract of rat anterior pituitary gland were injected daily for four days into hypophysectomized rats. On the fifth day thyroid weight and thyroid/serum iodide ratio (T/S) were measured. At low levels of stimulation, T/S was found to be a far more sensitive measure of thyroid activation than was thyroid weight change. These results are considered in relationship to the dissociation between T/S and thyroid size in rats with hypothalamic lesions.

In the presence of anterior hypothalamic damage thyroid growth response to propylthiouracil administration (1, 2, 3) or to partial thyroidectomy (4) is impaired or abolished. In contrast, the avidity of the gland for iodine, as measured by the thyroid/serum iodide ratio (T/S) increases to the usual degree. The dissociation between growth and functional response to goitrogen administration, which can also be demonstrated in mice bearing intra-ocular pituitary transplants (5), was at first interpreted by Greer as evidence for the existence of two kinds of TSH, one influencing growth, and the other function of the thyroid gland. This interesting suggestion stimulated a number of studies which have further clarified the effects of hypothalamic lesions on thyroid function (6, 7, 8), the action of propylthiouracil on the thyroid gland independent of TSH (9, 10) and the effects of low dosage of TSH on thyroid function and structure (11).

In a recent paper (12), Greer reported the results of giving graded doses of TSH to hypophysectomized mice and of graded doses of thyroxine to propylthiouracil treated mice and concluded that the dissociation between growth response and functional response could best be explained "by different thresholds of the individual processes to a single thyrotropin" rather than by postulating that "more than one thyrotropin is involved in their control."

In this laboratory, experiments similar to those of Greer have recently been completed with comparable findings. Since they were done in a different animal (rat instead of mouse) using a whole pituitary preparation instead of purified TSH, they are being presented.

METHODS

Hypophysectomized Holtzman rats[2] between 130 and 154 gm. in weight were used in this study. They were maintained on Purina laboratory chow with supplements of orange slices and bread and were housed in a controlled temperature room at $26 \pm 1°$ C. Animals were 14 days post-hypophysectomy at the time that assay was begun.

Donor pituitaries were obtained from male Holtzman rats weighing between 250 and 450 gm. The animals were killed with ether, the pituitaries removed quickly and the anterior lobe separated and placed immediately into the deep freeze. In order to prepare the extract, the frozen whole anterior pituitaries were pooled, homogenized in saline and refrozen in aliquots so that each daily dose could be thawed just before injection. Dilutions were made up so that each animal received 1.0 ml. of solution daily containing the appropriate fraction of whole pituitary gland. Groups of hypophysectomized rats were given extracts equivalent to 0.25, 0.5 and 1.0 pituitary per day for four days. Twenty-four hours following the last injection, the thyroid weight and thyroid/serum iodide ratio was measured using a technique modified from that of VanderLaan and Greer (13).

RESULTS

Progressive increase in both thyroid weight and T/S followed the administration of graded doses of pituitary extract (Fig. 1). These two parameters of thyroid stimulation

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[2] Hormone Assay Laboratories, Chicago, Illinois.

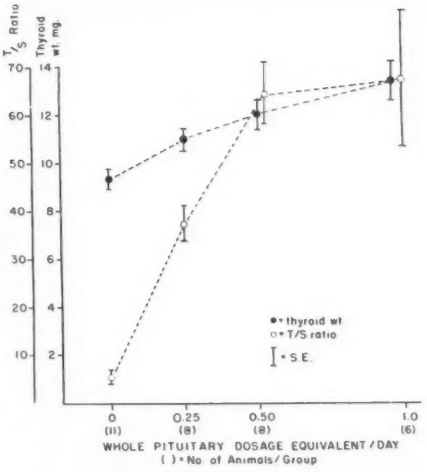

FIG. 1. Effects of graded doses of anterior pituitary extract on thyroid size and thyroid/serum iodide (T/S) ratio in hypophysectomized rats. Dosage of extract corresponds to the fraction of whole gland administered each day for four days.

did not rise in a parallel fashion however. In going from levels of no stimulation to 0.25 and 0.50 pituitary equivalents per day, T/S increased to a greater degree than did weight; between 0.5 and 1.0 equivalents per day, thyroid weight continued to rise while T/S ratio tended toward a plateau. At low levels of TSH stimulation therefore T/S ratio responds to a greater extent than does thyroid weight.

DISCUSSION

These results are compatible with Greer's observation that I^{131} uptake gives a steeper dose-response curve to TSH stimulation than does thyroid size in the mouse (12). They are also in harmony with the finding of the Bogdanoves (11) that in PTU fed rats there is a dissociation between T/S ratio and thyroid growth response at low levels of stimulation. The conclusion is warranted that these parameters of gland activation respond in a quantitatively different fashion to TSH and that dissociation of growth and function alone is not evidence for the existence of more than one kind of TSH.

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STRESS-INDUCED ADRENAL ASCORBIC ACID DEPLETION IN THE CAT[1]

ABSTRACT

A significant adrenal ascorbic acid depletion results from the stress of acute unilateral adrenalectomy in the cat. Hydrocortisone pretreatment blocks this response, and exogenous ACTH overcomes corticoid blockade, as in the rat. The cat does not demonstrate as unequivocally as other species "compensatory" adrenal hypertrophy in response to chronic unilateral adrenalectomy.

In the course of studies on effects of chronic limbic lesions in the cat (1) it was considered desirable to include a measure of the pituitary adrenocortical response to stress, since it is known that stimulation in limbic areas induces a rise in blood corticosteroids (2).

Adrenal ascorbic acid depletion following stress has been well validated as a measure of acute ACTH release in the rat and guinea pig (3), but has not been studied thoroughly in other species. Dohan and Lukens (4) demonstrated a 20% depletion of adrenal ascorbic acid in the cat following intraperitoneal glucose, assumed to be an indication of endogenous ACTH release. In the study described below, the ability of acute unilateral adrenalectomy to cause adrenal ascorbic acid depletion was tested in normal cats. The capacity of corticoid pre-treatment to block this response (5, 6) and of exogenous ACTH to overcome the blockade (6) were also tested. In addition, the effect of chronic unilateral adrenalectomy on the weight of the remaining adrenal was investigated, since Lobban has claimed (7) that the cat fails to demonstrate "compensatory" adrenal hypertrophy, in contrast to the rat (8) and dog (9).

MATERIALS AND METHODS

Thirty healthy cats of both sexes were used in the following experiments. Animals were fasted for 16 hours before pentobarbital (30 mg./kg. I.P.) was given.

Eighteen untreated cats were anesthetized and the first adrenal (usually the left) removed as quickly as possible (within 15 minutes for 16 cats). In 2 cats the second adrenal was removed within 2 or 20 minutes after the first, but in 16 animals the second adrenal was not removed until at least 45 minutes had elapsed. Each adrenal was weighed and the ascorbic acid content measured (10).

Six cats were pre-treated twice daily with 20 mg. doses of compound F intramuscularly for 2 days, a fifth dose being given on the morning of the third day. Several hours later the animals were subjected to the acute stress described above. Two of the cats were given 25 I.U. ACTH intravenously at the time of removal of the first adrenal.

Six cats had either the right or left adrenal removed under sterile conditions. Two or four weeks later the second adrenal was removed. Each adrenal was weighed.

RESULTS

The data on adrenal weight and ascorbic acid concentration and depletion on all acutely stressed cats are summarized in Table 1. The adrenal weight to body weight ratio the ascorbic acid concentration in the first adrenal, and the percentage depletion do not

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TABLE 1. ADRENAL WEIGHT AND ASCORBIC ACID CONCENTRATION AND DEPLETION TO ACUTE UNILATERAL ADRENALECTOMY IN CATS

Group	Sex	N	B.W., kg.	Adrenal wt., mg.		Tot. A.W., mg.	Adrenal ascorbic acid (first), μg./mg.	Interval between adrenal removals, min.	% ascorbic acid depletion*
				Left	Right	B.W., kg.			
Untreated	M	9	2.94 ± .68	163.4 ±27.2	173.3 ±49.5	117.1 ±25.2	0.98 ±0.24	56 ± 7	15.8 ±10.4
Untreated	F	8	2.35 ± .42	143.0 ±26.3	143.6 ±34.5	123.1 ±23.2	0.75 ±0.31	46 ±11	18.0 ±11.4
Untreated	F	1	2.01	96.0	116.9	105.8	0.91	2	10.4
5×20 mg. F	2M 2F	4	2.10 ± .53	217.2 ±93.4	207.9 ±77.8	217.3 ±105.4	0.76 ±0.27	50 ± 9	3.2 ±10.8
5×20 mg. F +25 c.c.ACTH	F F	1 1	2.98 2.10	256.8 147.5	246.7 133.5	169.3 134.0	0.64 0.61	50 53	27.3 35.7

± Standard Deviation.
* $\frac{\text{First, } \mu\text{g./mg.} - \text{second, } \mu\text{g./mg.}}{\text{First, } \mu\text{g./mg.}} \times 100$

differ significantly between male and female cats. No significant differences in weight were demonstrated between right and left adrenal. A significant ($P < .01$) ascorbic acid depletion occurred in both sexes, 18.0% ±4.0 (S.E.) and 15.8% ±3.5 (S.E.). However, 3 of the males and 2 of the females had less than 10% depletion. The cat with virtually simultaneous adrenal removals showed 10.4% depletion.

Hydrocortisone pre-treatment blocked the depletion of ascorbic acid (Table 1), while intravenously administered ACTH induced 27.3% and 35.7% depletion in 2 cats. Three of the 4 females receiving hydrocortisone showed remarkably increased adrenal weights.

The results of chronic adrenalectomy are shown in Table 2. The average difference in adrenal weight (+16.0 mg.) is not significantly greater than the standard error (Table 2), which is large owing to the wide range of responses. Three of the six cats showed a weight difference between the two adrenals which is of the order of magnitude of the hypertrophy seen in the rat (8) and dog (9).

DISCUSSION

Qualitatively speaking, the adrenal ascorbic acid in the cat responds similarly to that in the rat, since stress lowers the concentration, hydrocortisone blocks this effect and ACTH overcomes corticoid blockade. The initial concentration in the adrenal is, however, only one-quarter the concentration in the rat adrenal (6). The depletion of ascorbic acid in the second adrenal, while significant, was only 16% in the cat (Table 1),

TABLE 2. EFFECT OF CHRONIC UNILATERAL ADRENALECTOMY ON WEIGHT OF REMAINING ADRENAL IN THE CAT

Cat #	Sex	Interval between adrenal removals	B.W., kg.		Adrenal wt., mg.		Second A.W.—First A.W., mg.
			Initial	Terminal	First	Second	
1	F	4 wks.	1.87	1.79	134.3 (R)	137.1 (L)	+ 2.8
2	F	4 wks.	2.44	2.44	147.1 (L)	201.2 (R)	+54.1
3	F	2 wks.	2.01	2.32	129.2 (L)	144.5 (R)	+15.3
4	F	2 wks.	2.04	2.38	139.8 (R)	141.5 (L)	+ 1.7
5	M	2 wks.	3.09	2.78	151.5 (R)	176.3 (L)	+24.8
6	M	2 wks.	3.49	3.52	175.5 (L)	172.8 (R)	− 2.7

Average = +16.0
S.E. = ± 8.7

whereas unilateral adrenalectomy in the rat gives at least 30% depletion in comparison to unoperated controls (5). It is possible that the first adrenal in the cat was already partially depleted due to anesthesia and laparotomy (8). If this is so, then hydrocortisone failed to block this depletion (Table 1).

The failure of hydrocortisone to produce atrophy is probably related to the short duration of treatment. Since there were no sham-injected controls, it is not known whether the hypertrophy in three cats (Table 1) is due to a non-specific injection stress.

The data in Table 2 concerning "compensatory" adrenal hypertrophy are equivocal, leaving open the question of whether or not the cat demonstrates this phenomenon (7). Since hypophysectomy induces adrenal atrophy, and pituitary extract or stress causes hypertrophy in the cat (4), one would expect the usual hypertrophy in response to lowered circulating corticoids. It is possible that hyperactivity of the remaining adrenal cortex can occur in the cat without always being reflected in hypertrophy.

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Addendum

Since submission of the manuscript, Elton, *et al.*, (*Endocrinology*, **65:** 152, 1959) have reported no significant adrenal ascorbic acid depletion to cold exposure or intramuscular ACTH in the cat, in contrast to present results. The apparent discrepancy may be due to our use of the first adrenal as a control value in each cat, eliminating to some extent variability between animals.

EFFECT OF THYROXINE AND RELATED COMPOUNDS ON MONOAMINE OXIDASE ACTIVITY[1]

ABSTRACT

The activity of monoamine oxidase (MO) was studied in the liver, heart and brain of rats. The specific activity of heart was found to be 1.5 μmoles tyramine consumed/mg.N/hr.; that of brain 0.9, as compared to about 6.0 for liver. After a daily injection of thyroxine, triiodothyronine and triiodothyroacetic acid, on the 11th day the MO activity of liver was found to be decreased by about 50%. O-Methylthyroxine and thyroxamine caused a decrease of 30 and 25%, respectively. No significant changes in MO activity were noted in heart and brain.

In a recent paper (1) we reported that monoamine oxidase (MO) activity is lower than normal in the livers of rats fed desiccated thyroid. The present report describes the effect of injected thyroxine and some structurally related compounds on the activity of MO in several tissues.

MATERIALS AND METHODS

Male rats of Sprague-Dawley strain, 200–225 gm. were subcutaneously injected with the following compounds: Na-(L)-thyroxine pentahydrate (Elthrin; Smith, Kline and French); 3,3',5-triiodo-(L)-thyronine (Smith, Kline and French); 3,3',5-triiodothyroacetic acid (supplied kindly by Dr. Rosalind Pitt-Rivers); O-methyl-(D,L)-thyroxine and thyroxamine (prepared in this laboratory). The compounds were dissolved in a mixture of water and propylene glycol (1:1), buffered at pH 7–7.2, except the insoluble O-methyl-thyroxine and thyroxamine which were administered in a suspension of the same solvents. Control animals received injections of the solvents.

The injections were performed daily for 10 days. The dosage of thyroxine was 2.5 μg./gm. body wt. daily. Thyroxamine was given at twice this level and the other compounds were administered in doses approximately biologically equivalent to that of thyroxine. The rats were killed by decapitation on the 11th day and their liver, heart and brain were immediately prepared for enzymatic assay.

Mitochondria from liver and heart were prepared by the methods of Schneider (2) and Plaut (3), respectively. One ml. of the mitochondrial suspension in 0.25 M sucrose represented 1 gm. of the fresh tissue.

Brain tissue was used as homogenate, one ml. of the homogenate representing 1 gm. of the fresh tissue. The activity of monoamine oxidase was assayed measuring the amount of tyramine degraded after a 30-minute incubation period (1).

RESULTS

Our study of MO activity in thyroid hormone-treated rats is summarized in Table 1. All of the compounds tested were effective in lowering MO activity of the liver. The changes in MO activity of brain and heart were insignificant and inconsistent.

DISCUSSION

A previous paper from this laboratory (1) reported that feeding desiccated thyroid results in a significant depression of MO activity in the hepatic tissue of rat. This communication extends the above observation. The injection of various compounds which have thyroid hormone activity also resulted in a lowering of MO activity of liver. Doubling the dosage of thyroxine, triiodothyronine and triiodo-thyroacetic acid did not, however, cause a further significant decline in MO activity. The less pronounced effect of O-methyl-thyroxine and thyroxamine might be due to an incomplete absorption of these compounds, since they were injected in a suspension.

Thyroid hormones did not cause a significant decrease in the activity of MO in brain and heart. It may be that our assay is not sufficiently sensitive to detect change of MO in tissues initially low in this enzyme.

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TABLE 1. EFFECT OF THYROID HORMONES ON MO ACTIVITY IN VARIOUS TISSUES OF RAT

Compound injected	Dosage (μg./gm. of weight/day)	No. of animals	Specific activity* (average)		
			Liver	Heart	Brain
Thyroxine	2.5	4	2.6	1.0	0.9
Triiodothyronine	0.5	3	2.3	1.3	0.8
Triiodothyroacetic acid	5.0	4	2.6	1.7	1.0
O-Methyl-thyroxine	10.0	4	3.6	1.5	0.5
Thyroxamine	5.0	2	4.1	1.5	0.8
Controls	—	6	5.4	1.8	1.0

* μmoles tyramine consumed/mg. N/hour.

It appears from this preliminary work that different thyroid hormones exert a similar effect on MO in the liver.

The mechanism of thyroid hormone causing a decrease in hepatic MO activity is still unknown.

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EVIDENCE FOR LIVER INACTIVATION OF PARATHYROID HORMONE[1]

ABSTRACT

Parathyroid glands auto-transplanted to the hepatic portal circulation (spleen) failed to show hypertrophic changes. However, they were unable to maintain calcium levels of peritoneal lavage fluid equal to those held by normal controls or animals with auto-transplants in the peripheral circulation (testis). It is concluded that the lessened capacity of spleen-implanted parathyroids to mobilize calcium is due to partial inactivation of the hormone during its passage through the liver.

A short *in vivo* existence for parathyroid hormone has been indicated by the experiments of Munson (1) and Aurbach (2), although its metabolic fate has not been determined. Liver inactivation of estrogenic substances (3–5) and of progesterone (6–9) has been well established. If proteinaceous parathyroid hormone were also inactivated during passage through the liver, it was thought that normally operating physiological stimuli might be sufficient to produce a compensatory hypertrophy of parathyroid glands transplanted to the hepatic portal circulation (spleen). However, the histological appearance and estimated size of the implants after two weeks, or even after two months, proved to be essentially normal. These preliminary experiments indicated that any destruction of the hormone by the liver was insufficient under normal physiological conditions to initiate any major compensatory changes.

A more sensitive method has therefore been employed to determine if the spleen-im-

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planted parathyroid does have the functional capacity of intact parathyroid glands, or if any inactivation of the hormone occurs due to its secretion directly into the hepatic portal system. By the method of peritoneal lavage as employed in this laboratory (10) it is possible to determine the relative ability of physiological mechanisms, including those stimulated by parathyroid hormone, to replace diffusible calcium removed in the lavage fluid (11). By imposing this calcium stress, more minute differences in hormone titre should be detected.

MATERIALS AND METHODS

The animals used in these experiments were male Holtzman rats weighing 200–250 gm., and maintained on standard laboratory diet.

Auto-transplants of the parathyroid glands were made to the right tip of the spleen or just under the tunica albuginia of the testis. Both parathyroid glands were transplanted to the foreign site two weeks prior to the lavage experiment. Control rats were parathyroidectomized one week prior to the experiment.

Peritoneal lavage was performed as previously described (1), with the animals under light nembutal anesthesia. The lavage fluid consisted of 0.84% sodium chloride containing 1.5% glucose. Thirty ml. of fluid were placed in the peritoneal cavity, and replaced hourly with fresh fluid for 24 hours in the first experiment, and 7 hours in the second.

Calcium concentrations in the lavage fluid removed were determined with a flame spectrophotometer, after precipitating the calcium with ammonium oxalate and redissolving it in perchloric acid and isopropyl alcohol.

RESULTS AND DISCUSSION

In the first experiment, the lavage procedure was run continuously for 24 hours. The ability of animals with spleen-implanted parathyroids to supply calcium to the peritoneal fluid was compared to that of normal and parathyroidectomized rats. The data are summarized in Table 1. Animals with parathyroids implanted in the spleen maintained lavage fluid calciums significantly above those of parathyroidectomized rats, but only 87%

TABLE 1. RELATIVE ABILITY OF SPLEEN-IMPLANTED PARATHYROID GLANDS TO REPLACE CALCIUM REMOVED BY PERITONEAL LAVAGE

Treatment	No. rats	Calcium (mg.%) in lavage fluid, mean ± S.E.								
		Hour 2	Hour 5	Hour 8	Hour 11	Hour 14	Hour 17	Hour 20	Average	% of Normal
Normal	4	5.14 ± .25	4.54 ± .23	4.54 ± .10	4.47 ± .10	4.53 ± .28	4.73 ± .17	3.84 ± .10	4.68	100
Spleen-implanted	4	4.32 ± .19	4.01 ± .31	4.26 ± .17	3.80 ± .25	4.04 ± .08	4.08 ± .26	3.91 ± .29	4.06	87
Parathyroidectomized	4	2.30 ± .13	3.30 ± .32	3.13 ± .16	3.77 ± .28	3.15 ± .17	3.33 ± .13	3.30 ± .28	3.18	69

of the level held by parathyroid-intact controls. While it was obvious that the spleen-implanted parathyroids were actively secreting hormone, they demonstrated a reduced capacity to replenish the calcium removed in these test conditions.

To determine whether the lower calcium titre maintained by animals with spleen-implants was due to their location in the hepatic portal circulation, a second experiment was run. In addition to the three groups used earlier, a fourth group was added in which the parathyroids were implanted in the peripheral circulation (testis). Since the long lavage duration appeared unnecessary, this experiment was terminated after 7 hours. These data are summarized in Table 2. Calcium values of lavage fluid taken from animals with testes-implanted parathyroids were not statistically lower than normal control levels. However, again, the calcium levels from those animals with spleen implants were significantly lower than normal controls, but well above those from parathyroidectomized rats.

It is concluded that partial inactivation of parathyroid hormone during passage through the liver is responsible for the lessened capacity of spleen-implanted para-

TABLE 2. RELATIVE ABILITY OF TESTIS-IMPLANTED AND SPLEEN-IMPLANTED PARATHYROID GLANDS TO REPLACE CALCIUM REMOVED BY PERITONEAL LAVAGE

Treatment	No. rats	Calcium (mg. %) in lavage fluid, mean ±S.E.					
		Hour 1	Hour 3	Hour 5	Hour 7	Average	% of normal
Normal	4	4.33 ±.19	4.50 ±.26	5.23 ±.41	5.10 ±.25	4.79	100
Testis-implanted	4	4.18 ±.57	4.48 ±.26	4.68 ±.17	5.55 ±.37	4.72	98
Spleen-implanted	4	3.33 ±.26	3.74 ±.10	4.33 ±.10	4.60 ±.25	4.00	83
Parathyroidectomized	4	3.43 ±.28	3.45 ±.21	3.78 ±.14	3.90 ±.10	3.63	73

thyroid tissue to replace diffusible calcium removed by peritoneal lavage. The extent of the apparent inactivation is greater than anticipated from the lack of marked compensatory hypertrophy noted in histological sections. However, a number of authors have reported only slight regenerative capacity (12, 13) of this tissue after unilateral parathyroidectomy. These experiments bear out one of the difficulties in the study of parathyroid tissue; namely, obvious secretory changes can not as yet be detected by changes in the cytology of the tissue.

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THE EFFECT OF PROLACTIN ON THE XANTHINE OXIDIZING ACTIVITY OF PIGEON TISSUES[1]

ABSTRACT

The injection of prolactin into pigeons was found to lower the xanthine oxidizing activity of liver, kidney, pancreas and especially crop sac. Prolactin also decreased the amount of uric acid excreted.

There appears to be a factor in the pituitary gland of the rat, which suppresses the xanthine oxidizing activity of the liver, since Reid, O'Neal and Lewin (1) observed an increase in this activity following hypophysectomy. That the pituitary factor involved is not growth hormone is evident from the finding of these workers that injection of growth hormone into hypophysectomized rats did not lower the xanthine oxidizing activity; and since adrenalectomy of normal rats lowers the activity in the liver, the suppressing factor does not seem to be adrenocorticotrophin. One of the pituitary hormones which might be tested for this ability to suppress the level of xanthine oxidizing activity is prolactin. As a preliminary experiment in the study of this and related questions, the effect of prolactin on xanthine oxidizing activity was tested using normal pigeons, whose crop sacs are especially sensitive to the injection of prolactin (2).

EXPERIMENTAL

Silver King pigeons weighing about 500 gm. were used. Daily doses of 0.25 mg. prolactin were given for four days, and the birds were killed on the fifth day. The prolactin used was prepared by the procedure of Cole and Li (3).

After killing the birds by decapitation, tissues were quickly excised and chilled on ice. The weight of each tissue was determined, and then the tissue was minced with scissors and homogenized in 10 volumes of cold 0.05% KCl. The homogenization was carried out for 2–3 minutes in a glass homogenizer with "Teflon" pestle. In most cases the homogenate was centrifuged for 10 minutes at 4800×G in the cold, and the supernatant fluid used for assay. The whole homogenate was assayed on occasion and showed the same specific activity as the supernatant fluid.

Xanthine oxidizing activity was measured by the procedure of Kalkar (4) after confirming that the conditions suggested were optimal for activity measurements in the present case. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined respectively by the procedure of Siebert (5), and of Albaum and Umbreit (6) after the extraction of nucleic acids from the tissue according to the procedure of Greenbaum and Slater (7).

Excreta of the pigeons was collected on plastic pads which covered the floor of the cages. Uric acid was isolated from the excreta as described by Johnson and Johnson (8).

RESULTS AND DISCUSSION

It is apparent from the data presented in Table 1 that after the injection of prolactin into pigeons, the xanthine oxidizing activity per mg. of tissue was lowered in the case of liver, pancreas and kidney as well as in the case of crop sac. It is of interest that the crop sac whose growth is especially sensitive to prolactin, shows an especially large change in activity. The lowering of xanthine oxidizing activity in the tissues of pigeon by the injection of prolactin is reflected in the lowered production of uric acid; uric acid isolated from the excreta during the four days of injection was 0.34–0.42 gm. for each uninjected bird and 0.20–0.22 gm. for each bird receiving prolactin.

That the suppression of xanthine oxidizing activity is correlated with the accumulation of DNA and RNA in the crop sac is clear (see Table 2). Indeed, the correlation is maintained if the injections of prolactin are continued for a total of eight days in that the RNA, DNA and xanthine oxidizing levels remain essentially constant even though the crop sac increases in weight from 5 to 7 grams. The data also show, however, that no such correlation holds in the liver since there is no significant change in DNA content while the enzymic level in the liver was lowered in much the same way as in the case of

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TABLE 1. XANTHINE OXIDIZING ACTIVITY OF PIGEON TISSUES

Tissue	Activity*/gm. tissue		Total activity in tissue	
	Control pigeons	Pigeons receiving prolactin	Control pigeons	Pigeons receiving prolactin
Crop sac	25 ± 5**	7 ± 3	31	26
Liver	40 ± 2	15 ± 2	394	258
Kidney	90 ± 16	38 ± 7	335	238
Pancreas	70 ± 3	24 ± 1	175	114

Each figure represents the average of 7–8 birds.
* μg. xanthine oxidized/minute.
** Average deviation.

TABLE 2. NUCLEIC ACID CONTENT OF PIGEON TISSUES

Tissue or constituent	Amount in total tissue	
	Control pigeons	Injected pigeons
Crop sac		
Total weight	1.5 gm.	5.0 gm.
RNA	2.2 mg.	39.0 mg.
DNA	1.6 mg.	19.0 mg.
Liver		
Total weight	12 gm.	15.0 gm.
DNA	56 mg.	62.0 mg.

Each figure represents the average of 12 birds. Average deviation of the results was about 17% in all cases.

crop sac. Therefore, it does not appear that prolactin controls levels of DNA and RNA simply as a result of inhibition of purine catabolism, even though xanthine oxidase may play a key role in such catabolism (9). While these data do not permit conclusions regarding the mechanism of action of prolactin, they do suggest at least a partial explanation of the findings of Reid, O'Neal and Lewin (1) since on the basis of the present data, hypophysectomy would be expected to increase xanthine oxidizing activity by removing the source of prolactin.

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THE ENDOCRINE SOCIETY

1960 Annual Meeting

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday, and Saturday, June 9, 10, 11, 1960.

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10 at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel will hold 75 rooms each, for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12th for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12th on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond this date can be accepted directly by the hotels. Make your reservations now and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1st 1960 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Robert H. Williams, University of Washington School of Medicine, Seattle 5, Washington, not later than February 15, 1960. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.

2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words;
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)". Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)". The principal degree, e.g., M.D., of each author should be written after his name.

   Line 3. Institution of origin and city in which institution is located.

4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.

5. Authors are also to enclose 3×5 filing cards, one for each author, giving his name and initials in the upper right hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors and mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.

6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Williams.

7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I.L. Chaikoff in 1958 and Dr. Wilbur W. Swingle in 1959. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, and 1959 Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology.

Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1959.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the secretary of the Society. They should be submitted by October 15, 1959.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

ANNOUNCEMENTS

1960 LAURENTIAN HORMONE CONFERENCE

THE 1960 Laurentian Hormone Conference of the AAAS will be held at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the week September 4–9. Investigators interested in attending this Conference should make application to the Committee on Arrangements of the Laurentian Hormone Conference, 222 Maple Ave., Shrewsbury, Massachusetts, at an early date and in any event no later than May 13, 1960. A conference rate of $14.00 per day per person is extended to all invited participants. Since the number of our participants is necessarily limited by available accommodations, all applications are screened and invitations to attend are issued by the Committee on Arrangements by the second week in June. The following program has been arranged.

1960 LAURENTIAN HORMONE CONFERENCE PROGRAM

SEPTEMBER 4 THROUGH 9

I. TESTES FUNCTION

Function of normal and abnormal testicular interstitial cells in the mouse.
Robert A. Huseby, Univ. of Colorado, Denver; Oscar Dominguez and Leo T. Samuels, Univ. of Utah, Salt Lake City
Sunday evening, September 4

Genetic aspects of male hypogonadism.
H. Nowakowski and W. Lenz, Univ.-Klinik und Poliklinik, Hamburg-Eppendorf, Germany
Monday morning, September 5

II. HORMONES AND REPRODUCTION

Erucic acid and reproduction.
Robert L. Noble, Univ. of Western Ontario, London, Canada
Monday morning, September 5

Comparative physiology of reproduction and endocrinology of female domestic animals.
A. V. Nalbandov, Univ. of Illinois, Urbana, Illinois
Monday evening, September 5

Estrogen metabolism in the human fetus and new-born.
E. Diczfalusy, Karolinska Institut, Stockholm, Sweden
Tuesday morning, September 6

Placental transmission of hormones.
Claude Migeon, Johns Hopkins Hospital, Baltimore; Jean Bertrand, Hopital Edourard Herriot, Lyon, France; Carl Gemzell, Karolinska Institut, Stockholm, Sweden
Tuesday morning, September 6

The metabolic influence of progesterone in man.
Richard L. Landau, Univ. of Chicago, Chicago, Illinois
Tuesday evening, September 6

III. Aldosterone

Mechanisms regulating the secretion and metabolism of aldosterone.
James O. Davis, National Heart Institute, Bethesda, Maryland
Wednesday morning, September 7

The role of aldosterone in experimental nephrosis in the rat.
Claude J. P. Giroud, Montreal Children's Hospital; Norman Kalant, Jewish General Hospital, Montreal; R. H. Despointes, and D. Das Gupta, Montreal Children's Hospital, Montreal
Wednesday morning, September 7

Clinical endocrinology of aldosterone.
Jerome Conn, University Hospitals, Ann Arbor, Michigan
Thursday morning, September 8

IV. Hormones and Electrolyte Metabolism

Comparative physiology and pharmacology of the neurohypophysis.
W. H. Sawyer, Columbia University, College of Physicians and Surgeons, New York
Thursday morning, September 8

The effects of neurohypophyseal hormone on permeability and transport in living membrane.
Alexander Leaf and Richard M. Hays, Massachusetts General Hospital, Boston
Thursday evening, September 8

V. Hormones and Organic Metabolism

The regulation of glucose uptake in muscle by hormones and other factors.
C. R. Park, Vanderbilt University, Nashville, Tennessee
Friday morning, September 9

The direct action of hormones on liver metabolism.
Leon L. Miller, University of Rochester, New York
Friday morning, September 9

JAHRESTAGUNG DER INTERNATIONAL SOCIETY OF NEUROVEGETATIVE RESEARCH: GRÜNDUNGS-TAGUNG DER DEUTSCHEN NEURO-VEGETATIVEN GESELLSCHAFT

VOM 29. BIS 31. JULI 1960
IN FREUDENSTADT/SCHWARZWALD

Ehren-Präsidium: PROF. DR. W. R. HESS, Zürich
Tagungs-Leitung: PROF. DR. A. STURM, Wuppertal UND PROF. DR. E. TONUTTI, Tübingen

X. NEUROVEGETATIVES SYMPOSION

Vorläufiges Programm der wissenschaftlichen Sitzungen am 29. VII., vorm., nachm. und 30. VII. vorm.:

1. Hauptthema: Hypothalamische Steuerung der ACTH-Sekretion

M. MOSINGER, Marseille (Institut d'Hygiène industrielle, de Médecine du Travail et de Médecine légale)
"Hypothalamus, ACTH et corticostéroides. Recherches expérimentales"
J. W. MASON AND E. SACHAR, Washington—USA (Walter Reed Army Institute of Research, Department of Neuroendocrinology)
"The role of Limbic-system structures in the regulation of ACTH-secretion"
S. OKINAKA, Tokyo—Japan (III. Med. Klinik, University of Tokyo)
"Die Steuerung der ACTH-Sekretion durch das Limbische System und den Hypothalamus"
S. KATSUKI, Fukuoka—Japan (Kyushu University)
"Central control of Adreno-cortical activities, with special reference to the functional localisation within the Hypothalamus"
E. TONUTTI, Tübingen, (Anatomisches Institut der Universität Tübingen)
"Lokalisation der ACTH-Steuerung im Hypothalamus"
R. GUILLEMIN AND A. V. SCHALLY (Department of Physiology, Baylor University College of Medicine, Houston, USA, and Laboratoire d'Endocrinologie Expérimentale, Collège de France, Paris)
"Zur Frage einer hypothalamischen Überträgersubstanz für Steuerung der ACTH-Sekretion"
F. HENI, Tübingen (Medizinische Universitäts-Klinik)
"Die Bewertung des Eosinophilentestes als Funktionsprobe des Hypophysen-Nebennierensystems"
L. WEISSBECKER, Karlsruhe (II. Medizinische Klinik)
"Die Bewertung des Corticoidnachweises in Harn und Blut"
Zur Diskussion vorgemerkt: A. LUNEDEI, Florenz UND E. ANDERSON, Bethesda, USA
Außerhalb des Hauptthemas: J. A. KAPPERS, Groningen (Anat.-Embryol-Labor der Universität Groningen)
"Die Innervation der Epiphysis cerebri der Ratte"

Am 30. VII., nachm. und 31. VII., vorm.:

2. Hauptthema: Die cerebralen vegetativen Anfälle

F. BROSER, Würzburg (Universitäts-Nervenklinik) Übersichtsreferat

H. GASTAUT, Marseille (Institut d'Anatomie Pathologique, International Federoatin of Societies for Electroencephalography and Clinical Neurophysiology)
"Le diagnostic polygraphique (E.E.G., E.K.G. et enregistrement tensionnel) entre les crises épileptiques et les crises ischémiques partielles) ou généralisées"
G. BELLONI, Padua (Clinica delle Malattie Nervose e Mentali)
"Zur vegetativen Epilepsie"
W. SCHULTE, Gütersloh (Landesheil- und Krankenanstalt)
"Syncopale Anfälle"
R. MEYER-MICKELEIT, Freiburg i. Br. (Abteilung für Klinische Neurophysiologie der Universität Freiburg)
"Vegetative Symptome bei der temporalen Epilepsie"
P. GLOOR, Montreal—Kanada (Neurological Institute, McGill University, Montreal)
"The relationship of the temporal lobe with thalamus and hypothalamus"
S. SHIMODA (Tottori University, Japan)
"The Clinical and electro-encephalic study of diencephalic epilepsy"
W. BIRKMAYER, Wien (Neurologische Abteilung der Stadt Wien-Lainz)
"Mittelhirn-Anfälle"
W. SCHOBER, Wien.
"Zur Frage posttraumatischer diencephaler Anfälle"
K. J. ZÜLCH, R. CH. BEHREND UND R. LOHMANN, Köln (Abt. für Allgemeine Neurologie am Max-Planck-Institut für Hirnforschung)
"Zur Frage des Angiospasmus bei der Entstehung der Migraine und anderer Hirngefäßsyndrome"
H. JANTZ, Frankfurt (Main) (Universitäts-Nervenklinik)
"Pharmakologische Beeinflußbarkeit der cerebralen vegetativen Anfälle"
Zur Diskussion vorgemerkt: H. PETTE, Hamburg UND G. BODECHTEL, München

"Communications regarding the meeting should be directed to Professor Dr. A. Sturm, Medizinische u. Nervenklinik der Städtischen Kranken-Anstalten, Wuppertal-Barmen, Germany."

SYMPOSIUM ON UTERINE IMPLANTATION OF OVA

A SYMPOSIUM devoted entirely to problems relative to the uterine implantation of ova, in animals and in the human being, will be held in Brussels, Belgium, June 24–26, 1960, just before the First International Congress of Endocrinology in Copenhagen.

It is being organized jointly by the Société Nationale Française pour l'Etude de la Stérilité et de la Fécondité (Président: Professeur P. Funck-Brentano) and the Belgian Section of the International Fertility Association.

All interested persons are invited to attend. The working languages will be English and French, with simultaneous translation.

Registration: Dr. P. Wilkin, 75, rue Général Lotz, Brussels, Belgium.

Registration fees (volumes of the Proceedings included): 20 dollars.

Scientific information: Dr. J. Ferin, 42 Beukenlaan, Heverlee-Louvain, Belgium.

PROGRAM OF THE MAIN LECTURES

G. Mayer Bordeaux (France)	Morphologie et physiologie comparées de l'ovo-implantation
R. Canivenc Bordeaux (France)	La nidation différée
M. Shelesnyak Rehovoth (Israel)	The mechanisms of decidualisation: experimental study
B. Böving Baltimore (USA)	Interplay of mechanisms in rabbit blastocyst implantation
C. Lutwak-Mann Cambridge (England)	Biochemicals aspects of ovum implantation
E. C. Amoroso London (England)	Factors of early embryonic death
E. Ramsey Baltimore (USA)	The normal nidation in the rhesus monkey (Macaca Mulatta)
A. T. Hertig Boston (USA)	The nidation of normal and abnormal fertilized human ova
R. Cartier, F. Moricard et R. Moricard Paris (France)	Problèmes posés par l'activité sécrétoire de l'épithélium cylindrique utérin et un oedème du chorion cytogène précédant la nidation de l'oeuf
M. Gaudefroy Lille (France)	Exploration cyto-hormonale de l'endomètre après nidation récente
E. Bontke Cologne (Germany)	Histochemistry of premenstrual and gravid endometrium.
H. Hamperl et G. Hellweg Bonn (Germany)	Granular stroma cells of premenstrual and gravid endometrium
P. Wilkin Bruxelles (Belgique)	L'appareil vasculaire dans l'endomètre prémenstruel et gravidique
R. Palmer Paris (France)	Le comportement musculaire de l'utérus et de ses sphincters à l'époque de la nidation

S. Aschheim et P. Aschheim Paris (France)	La diagnostic hormonal de la nidation récente
J. Ferin et A. Van Gansewinkel Louvain (Belgique)	Les hémorragies de la période de nidation
L. Snaith Newcastle-on-Tyne (England)	The early spontaneous abortions
G. I. M. Swyer London (England)	Therapeutics problems of early abortions

TRAINING PROGRAM FOR STEROID BIOCHEMISTRY

The Worcester Foundation for Experimental Biology, Shrewsbury Massachusetts, with The Department of Chemistry, Clark University, Worcester, Massachusetts, and The Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City, announce the 1960–1961 Session of the *Training Program for Steroid Biochemistry.*

To alleviate the shortage of qualified trained personnel in steroid research, a Training Program will be sponsored by the United States Public Health Service through the National Cancer Institute of the National Institutes of Health. The courses will stress both theoretical and practical aspects as well as specialized laboratory techniques in steroid research.

Post-doctoral candidates (M.D. or Ph.D.) selected for admission both at Worcester and Utah will receive stipends of $5500 for a one-year period. Pre-doctoral candidates (B.S., M.S., or equivalent) selected for admission (only at Worcester) will receive stipends of $1800 for a six-month period.

Send inquiries and requests for applications to:

Dr. Kristen Eik-Nes Department of Biochemistry College of Medicine University of Utah Salt Lake City, Utah	or	Dr. William R. Nes Department of Chemistry Clark University 950 Main Street Worcester, Massachusetts

Classes start October 1, 1960.

Final date for completion of application: June 1, 1960.

CORRECTION

In the note by H. C. Kwaan and H. J. Bartelstone, entitled "Corticotropin Release Following Injections of Minute Doses of Arginine Vasopressin into the Third Ventricle of the Dog" which appeared in the December 1959 issue of Endocrinology the following errors should be noted:

Page 984—3rd line of Addendum, 5 μμg. should read 5 mμg.

Page 985—Reference No. 15—the first name "van Dyke, H. B." should read "Miller, C. P."